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Abstract

Title of Dissertation:

Defective Priming of $CD4^+$ T Cell Responses During Pre-patent Schistosome Infection.

Christine E. Ferragine, Doctor of Philosophy, 2011

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Schistosomes are intravascular helminths that affect approximately 200 million people throughout the tropics and subtropics. An early Th1 response to schistosome worms is replaced at roughly 6 weeks post infection by a robust Th2 granulomatous response initiated by egg deposition. The host immune response against schistosome worms is critical in mediating the subsequent adaptive

response that controls egg-induced immuno-pathology and may have a role in host resistance and susceptibility to co-infections. Here we sought to investigate whether alterations in the innate immune response induced by schistosomes create an immuno-modulatory milieu permissive for parasite establishment and development. Our data show that pre-patent infection induces the expression of cytokines representative of Th1, Th2, and T regulatory responses and that, in addition to IFN-γ, $CD4^+$ T cells stimulated with worm antigen produce IL-4 and IL-10. We have demonstrated that pre-patent infection causes host T cell responses to polyclonal stimulation to become hyporesponsive. Although T regulatory cells play a role in immune regulation by producing the regulatory cytokine IL-10, they are not indicated as a significant source and do not function to control Th1 responses during pre-patent schistosome infection. We provide evidence that schistosome infection leads to a reduction in host T cell responses to non-parasite antigen and that this loss of responsiveness is due to an inability of innate APCs to stimulate T cell proliferation and cytokine production. Our analysis of the innate APC response implicates $CD11b^+$ mononuclear cells as the population of cells affected by this loss of T cell stimulatory capacity, where schistosome infection interferes with the ability of $CD11b^+$ cells to act as antigen presenting cells and stimulate T cell responses. The mechanism of this impairment is independent of many common regulatory methods, including the production of a diffusible inhibitor, but is dependent on cell contact and appears to in part be due to suppression of innate APC production of IL-12. Schistosome modulation of mononuclear cell function and T cell responsiveness is fascinating

as we have recently shown that schistosomes may manipulate these cells for their own advantage to facilitate proper development.

Defective Priming of $CD4^+$ T Cell Responses During Pre-patent Schistosome Infection

by

Christine E. Ferragine

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Dedication

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General Introduction

Schistosomiasis

History and Discovery

Schistosomiasis is a devastating disease caused by trematodes of the genus *Schistosoma.* Some of the earliest reports of schistosomiasis come from the ancient Egyptians, who marked a young man's transition into adulthood by the appearance of blood in the urine [1]. The symptoms detailed in these early writings are consistent with the disease known today to be caused by schistosomes. The first description of the organism responsible for schistosomiasis was by Theodore Bilharz, a German physician, in 1852; who identified adult worms and eggs in tissue and fecal samples [2]. The first published work on schistosomiasis in Brazil was by a Brazilian medical doctor named Pirajá da Silva [3], however the disease is commonly called bilharziasis after Theodore Bilharz. Once the organism's life cycle was fully elucidated, approximately 60 years after Bilharz's description in 1852, the scientific community began to investigate the pathogenesis and public health effect of infection [2].

Epidemiology

Despite roughly 160 years of research into the disease, schistosomiasis remains a major public health issue. It is estimated that there are approximately 200 million people infected in 74 endemic developing countries [4], with the majority, roughly 90%, in sub-Saharan Africa [5]. The largest number of cases

occurs in Nigeria, with around 29 million people infected [6]. Two thirds of all schistosomiasis cases are caused by infection with *S. haematobium*, leading to urinary tract disease [7]. The toll of infection on people living in endemic areas is significant, both physically and economically, and has recently been re-evaluated and increased to a disability-adjusted life year (DALY) that has been calculated to be 70 million per year [8]. The highest prevalence and intensity of infection is seen in school age children [6]. The disease in children is especially debilitating and can result in malnourishment and iron deficiency anemia that in turn leads to growth retardation [9]. Infected children may also display cognitive impairment and memory deficits that can be associated with developmental delays [10].

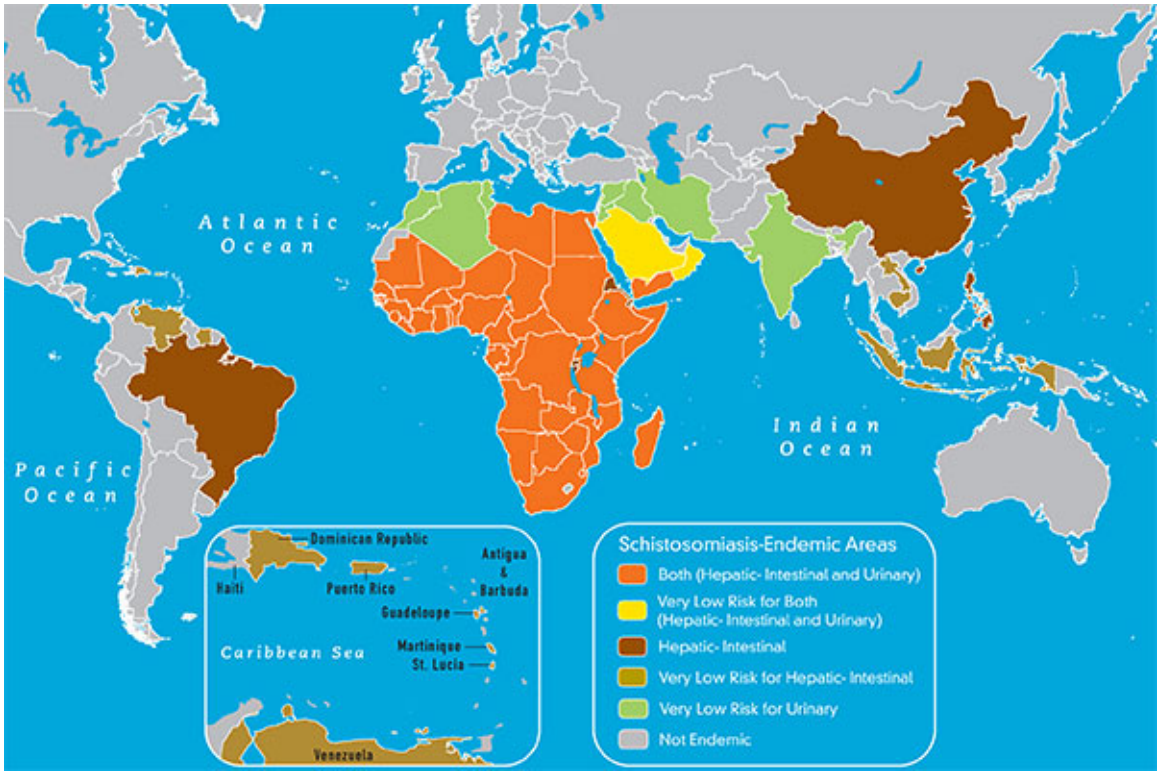
Geographical Distribution

There are three main species of schistosomes which cause disease in humans. The global distribution of schistosomiasis, including the types of disease found in endemic areas, can be seen in Figure 1. *S. mansoni* is found in Africa and Latin America, *S. japonicum* is found in China and the Philippines, and *S. haematobium* is found in Africa and the Middle East. The type of chronic disease caused by schistosome infection is dependent on where the adult worms reside anatomically. During infection with *S. mansoni* and *S. japonicum,* the adult worms reside in the mesenteric venules that drain the intestine. With both *S. mansoni* and *S. japonicum,* infection leads to hepatic and intestinal disease. In *S. haematobium* infection, the adult worms are found in the venous plexus of

the bladder and infection causes urinary disease [11, 12]. The geographical distribution of disease is influenced by the prevalence of competent snail vectors, whose habitat consists of shallow, fresh water.

Figure 1. Geographic distribution of Schistosomiasis

S. mansoni is found in Africa and Latin America, *S. japonicum* is found in China and the Philippines, and *S. haematobium* is found in Africa and the Middle East. Infection with *S. mansoni* and *S. japonicum* causes hepatic and intestinal disease and infection *S. haematobium* leads to urinary disease. Image from [12].


Atlantic Ocean
Pacific Ocean
Indian Ocean
Caribbean Sea
Dominican Republic
Haiti
Puerto Rico
Antigua & Barbuda
Guadeloupe
Martinique
St. Lucia
Venezuela
Schistosomiasis-Endemic Areas
Both (Hepatic- Intestinal and Urinary)
Very Low Risk for Both (Hepatic- Intestinal and Urinary)
Hepatic- Intestinal
Very Low Risk for Hepatic- Intestinal
Very Low Risk for Urinary
Not Endemic

Disease

There are two stages of disease in schistosomiasis, acute and chronic infection. Acute infection, also known as Katayama fever, is usually seen in travelers and aid workers who have visited endemic areas. This stage of illness is a hypersensitivity response that corresponds with the onset of schistosome egg-laying and is characterized by flu like symptoms, including fever and fatigue. Cercarial dermatitis is another form of illness seen during acute infection, usually in non-endemic areas. It is an IgE mediated hypersensitivity reaction that is often the result of infection with bird schistosome species that do not progress past the skin stage but can cause a pruritic, maculopapular rash [11]. Symptoms of acute infection are usually not seen in residents of endemic areas, likely because of desensitization developed in utero [13, 14] or failure to diagnose infection. The chronic stage of schistosome infection is variable depending on the location of adult worms within the host. It can consist of liver, bladder or intestinal disease and is a result of parasite eggs becoming trapped in the tissues of these organs. The most serious form of disease is hepatic splenic schistosomiasis. In rare cases this form of disease can lead to fatal complications such as severe bleeding from gastrointestinal varices.

Co-infections

Further complicating the forms of disease seen during chronic schistosome infection is the presence of co-infections. Co-infections commonly

seen with *S. mansoni* infection include hepatitis B or C virus (HBV or HCV) and human immunodeficiency virus (HIV) [15]. Co-infection with HBV or HCV is associated with accelerated progression of hepatitis and increased severity of illness [16]. The combination of infection with HBV and chronic *S. mansoni* may result in higher rates of hepatocellular carcinoma than with HBV infection alone [17]. During co-infection with HIV, studies have indicated that decreased levels of $CD4^+$ T cells correlate with lower egg excretion due to the requirement for a functional immune response to facilitate migration of eggs into the lumen of the gut; this reduction in egg excretion may lead to increased inflammation associated with those eggs that are retained [18]. Co-infection with genital schistosomiasis and HIV has also been shown to foster horizontal transmission of HIV [19].

Pathology

The pathology associated with schistosomiasis is mostly due to the eggs and not the adult worms. In fact, although the adult worms do cause cellular damage to host tissue and deposit their metabolic waste products [20, 21], they have not been shown to induce a considerable immunological response from the host. The eggs, on the other hand, elicit a profound immuno-pathological response that is governed by a $CD4^+$ T helper cell type 2 response [22]. Morbidity associated with chronic infection is a result of eggs becoming lodged in the tissue of the intestine, liver and other organs. These eggs then secrete

excretory/secretory (ES) products, which stimulate an inflammatory response[23]. The egg induced granulomatous response is an attempt by the host's immune system to wall off the egg, preventing secretion of toxic egg products into the host tissue, and restricting further damage to the host [23].

The granulomatous response is itself very harmful and causes extensive tissue damage. Although it can result in destruction of the egg, it also leads to fibrosis in host tissues. Tissue fibrosis is one of the more detrimental pathologies as it can restrict the hepatic vasculature and eventually lead to portal hypertension [1]. Most often, fatal complications associated with schistosomiasis are a result of portal hypertension causing bleeding from intestinal varices. During *S. mansoni* and *S. japonicum* infection the eggs are swept back into and become trapped in the tissue of the liver, leading to the induction of presinusoidal inflammation and periportal fibrosis, a condition called clay pipe stem fibrosis [24]. Another clinical manifestation of portal hypertension and egg-induced fibrosis is the enlargement of the spleen and liver; the classical sign of hepatosplenomegaly.

Genitourinary disease is associated with *S. haematobium* infection and is a result of eggs being deposited in the wall of the bladder and ureters. A significant percentage of women and men with genitourinary disease develop genital ulcers and lesions [8], which in women can lead to decreased reproductive health and infertility [25]. Chronic *S. haematobium* infection induces fibrosis and calcification of the bladder and ureters, which can be complicated by

secondary bacterial infections [26]. This form of disease results in hydroureter and hydronephrosis that in severe cases can lead to renal failure [11].

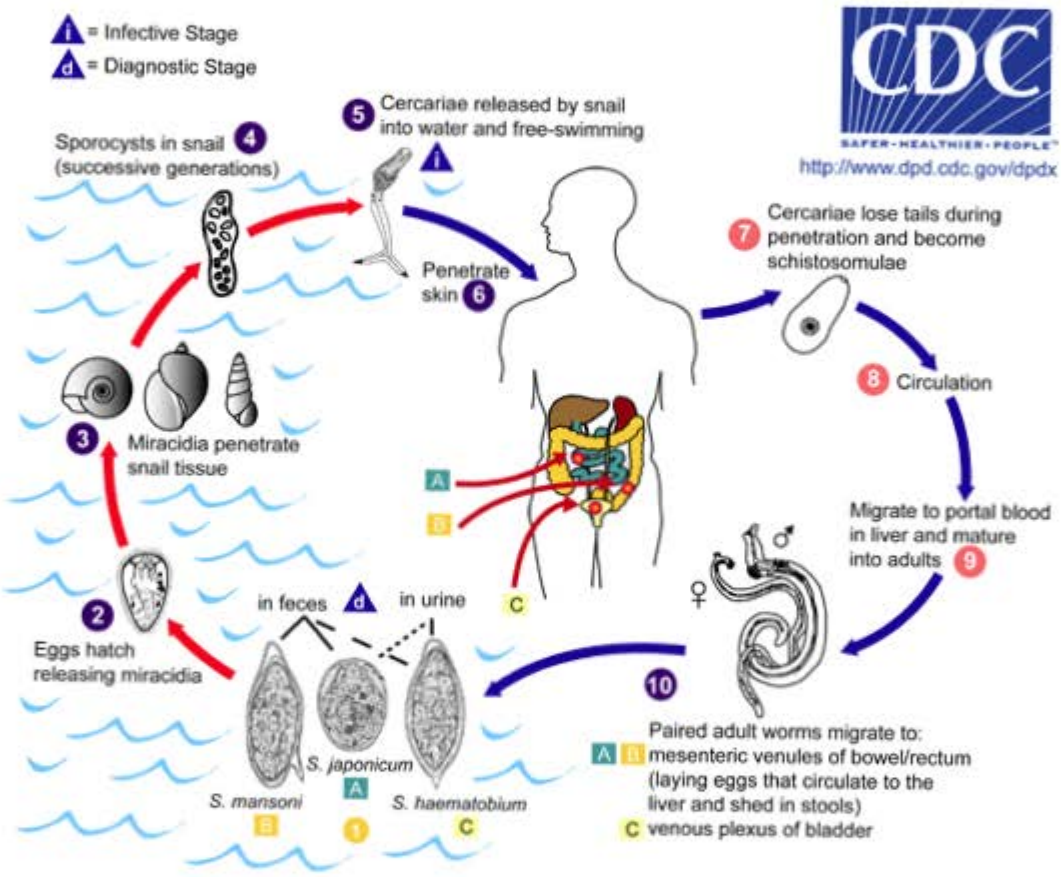
Life Cycle and Transmission

The schistosome life cycle, as shown in Figure 2, is complex and involves two hosts, the intermediate snail host and the definitive mammalian host. The infective stage for humans is the cercaria, a free-swimming organism found in fresh water. Transmission occurs via exposure to bodies of water that harbor competent snail vectors and contain infective cercariae. One infected snail can shed thousands of cercariae per day for several months [11]. The cercariae are phototropic and will congregate at the surface of water [1]. They are also attracted to certain chemicals in human skin and are able to penetrate intact human skin, usually through a hair follicle [27]. Upon penetration, cercariae shed their bifurcated tails and transform into schistosomula. The schistosomula migrate through the skin by secreting proteolytic enzymes and enter the circulation after a few days [11]. They migrate through the capillaries of the lung and after approximately one week they arrive at the portal venous system, where they mature into adult worms. Adult worms are dioecious and upon maturation will pair, with the smaller thread-like female residing within the gynacophoric canal of the larger male. Schistosomes mate in the liver then migrate to the mesenteric vasculature to produce eggs, four to six weeks post infection. The eggs can become trapped in tissue, leading to the pathology associated with

disease, or transit through the wall of the intestine, in the case of *S. mansoni and S. japonicum,* and are excreted from the host in the feces. In the case of S. *haematobium,* eggs transit through the wall of the bladder, and are excreted in the urine. The eggs hatch upon contact with fresh water and release miracidia. These miracidia then seek out their appropriate snail host, penetrate the snail and undergo several cycles of asexual reproduction within the snail. Cercariae are released from the snail four to six weeks after infection [11], in response to light, completing the life cycle.

Figure 2. Schistosomiasis life cycle

Schistosomiasis is caused by digenetic intravascular trematodes. The life cycle involves two hosts, an intermediate snail host and a definitive mammalian host. Image from [28].


i = Infective Stage
d = Diagnostic Stage
CDC
SAFER·HEALTHIER·PEOPLE™
http://www.dpd.cdc.gov/dpdx
5 Cercariae released by snail into water and free-swimming
Sporocysts in snail 4 (successive generations)
Penetrate skin 6
7 Cercariae lose tails during penetration and become schistosomulae
8 Circulation
3 Miracidia penetrate snail tissue
Migrate to portal blood in liver and mature into adults 9
2 Eggs hatch releasing miracidia
in feces
in urine
10
Paired adult worms migrate to:
A B mesenteric venules of bowel/rectum (laying eggs that circulate to the liver and shed in stools)
C venous plexus of bladder
S. japonicum
S. mansoni
S. haematobium

Burden of Disease

The study of schistosome infection is relevant to people living in endemic areas as the burden of infection is physically significant. Schistosome worms can survive in the host for 5-10 years [29], with the female worm producing hundreds to thousands of eggs per day, depending on the species, and as such, the burden on the individual host is tremendous [11]. Residents of endemic areas are constantly exposed to infection as part of their day-to-day lives [30]. Approximately 76% of the population in Sub-Saharan Africa lives near bodies of water, such as rivers and lakes, that contain snail intermediate hosts [31], making it difficult, if not impossible, to prevent re-infection. In addition, there is no evidence for the development of protective or sterilizing immunity to natural schistosome infection [32]. In endemic communities, infection levels peak in childhood at around 10 years of age, then decline rapidly over time to low levels in adulthood [33]. This infection pattern may indicate that the slow development of age dependent acquired immunity to schistosome infection relies on exposure to antigens from dying worms. There is evidence of acquired immunity to re-infection in people who have undergone several cycles of drug cure and re-infection [34], but this is dependent on the immunologic status of the individual and their infection history.

Treatment

Drug Treatment

Praziquantel, the mainstay of treatment for schistosomiasis, was developed in the mid-1970's and its safety and efficacy has contributed to its widespread use [35]. Praziquantel is effective against all schistosome species and, although its exact mechanism of action on adult worms is unknown, it is believed to work by paralyzing schistosomes and damaging the tegument [36, 37]. Resistance is especially concerning in the case of schistosomiasis because there is currently one effective drug treatment, praziquantel.

Resistance

There are reports of resistance in experimental, laboratory infections [38], and more relevantly, in strains isolated from several endemic areas [39, 40]. Strains of *S. mansoni* isolated from endemic areas have shown lower susceptibility to drug treatment [41]. The trend of decreased susceptibility could lead to increased resistance to drug treatment. Praziquantel remains an effective treatment for schistosomiasis, although there is significant evidence highlighting the potential for resistance [34]. However, there are limitations to chemotherapeutic control, including the expense of the drug itself, the difficulty in delivering it to endemic areas, and the fact that drug treatment does not prevent re-infection. The control of zoonotic transmission adds yet another level of

complexity to disease control. These factors highlight why schistosomiasis remains a major public health issue in spite of control initiatives.

Vaccine Strategies

Vaccine strategies have been developed in concert with chemotherapeutic methods in an effort to control for schistosomiasis. Attempts to develop a vaccine for schistosomiasis have been varied and extensive, yet no practical vaccine with real protective efficacy has been created [42]. This process has been made exceedingly difficult due to the limitation that schistosome infection does not result in protective immunity. It is even further complicated by the schistosome worm's extensive arsenal of immune evasion strategies. One key component to vaccine development is the understanding of the immune response to schistosomiasis. Methods of vaccination have been targeted against initial infection or parasite fecundity, where anti-fecundity vaccines could be beneficial to the host by to reducing parasite egg production and therefore pathology. The mainstay of assessing the efficacy of a vaccine is the reduction in worm number. The target of a protective immune response will likely be the migrating schistosomulum [43]. Some of the earliest schistosome vaccines investigating the injection of mice with worm extracts, then challenging them with cercariae, achieved inconsistent and small reductions in worm burden [44, 45]. Another investigation examined the role of concomitant immunity [46], where the thought was that an existing chronic infection could protect against secondary infection [47], however this vaccination strategy did not come to fruition.

A vaccination method that remains at the core of many strategies employed today is the attenuated cercarial vaccine [48]. Studies utilizing this method of vaccination have attained levels of protection from 80 to 90% [49, 50]. The critical aspect of this form of vaccine is the abbreviated migration of attenuated cercaria through the skin [51], where some parasites survive and enter the skin draining lymph node (sdLN) and elicit an immune response that is both stronger and different than that directed at non-attenuated parasites. Importantly, this vaccine model has led to the identification of potential effector mechanisms which may be critical for the development of an effective immune response [52]. Some of these mechanisms include innate immune cell activation in the skin [53], priming of a Th1 type response in the sdLN [54], and antigen specific $CD4^+$ T cell recruitment from the lungs to the circulation [55]. It appears that the protective immunity afforded by the attenuated cercarial vaccine is mediated by acquired immune mechanisms [56].

The hurdle to overcome in using the knowledge garnered from research on the attenuated cercarial vaccine is identifying antigens that mediate protective immunity. Several different approaches have been employed, with limited success, to identify protective schistosome antigens [57]; i.e. crude extracts [58], monoclonal antibodies [59-61], anti-idiotypes [62], expression library screening [63, 64], and identifying candidates based on immunogenic potential [65]. Despite all of these attempts to identify a vaccine candidate, only one antigen has entered clinical trials, the 28-kDa glutathione s-transferase (GST) from *S. haematobium* [66]. The use of recombinant antigen vaccines has not attained

the level of protection that the attenuated cercarial vaccine is able to achieve. This shortfall may be due to improper formulation, where the co-administration of recombinant interleukin-12 (IL-12) along with the attenuated cercarial vaccine was able to increase the efficacy of the vaccine [49]. The lack of success with vaccine strategies that have been employed to date may also be due to an incorrect delivery system or the antigens included in the vaccine may not elicit the type of immunological response that is required for protection. Going forward, the research community is focused on using new methods and techniques, including DNA microarray profiling, proteomics, glycomics, and immunomics, along with newly available data from the recently published schistosome genome [67], as well as the transcriptome [68] and proteome, to search for potential vaccine candidates. One of the most promising recent advances is the application of RNA interference to schistosomes [69, 70]. These types of studies will afford the ability to target specific genes and proteins and determine if they are essential for survival and fecundity.

Immune Evasion by the Parasite

The crucial obstacle in attempting the formidable task of searching for a schistosomiasis vaccine is the fact that schistosomes have developed in concert with the host, and the host's immune response, for millions of years [71]. This co-evolution has presumably armed schistosomes with a variety of defenses that enable them to persist in the face of the host's immune system attacks and

survive for long periods of time [72]. One putative mechanism of evasion is the masking of the schistosome surface by the adsorption of host proteins onto the worm's tegument, so that the schistosome is not recognized as foreign by the host [73]. For example, schistosomes have been shown to acquire host immunoglobulins [74] and major histocompatibility complex (MHC) proteins [75]. Another method of evasion is the suppression of the host's immune response. Schistosome worms appear to be able to utilize the host's regulatory immune response for their own advantage by inducing $CD4^{+}CD25^{+}$ Treg cells, and possibly the production of IL-10, to facilitate their escape from a protective Th1 immune response [76].

The immunological interaction between host and schistosome has developed to benefit the host by preventing severe pathology, but also has been alluded to benefit the parasite by facilitating survival [57, 77]. Schistosomes can survive in the host for prolonged periods without causing severe disease, with a very small percentage of infected individuals displaying the most serious forms of illness [78]. Studies in endemic areas, as well as animal experiments, have led to the hypothesis that the immune response to infection, and so the degree of disease severity, is governed by host genetics, infection intensity and the presence of co-infections [23].

Immune Response to Schistosome Infection

Overview

Models of the immune response to schistosome infection posit that a weak Th1 response is present during approximately the first six weeks of infection that is targeted against the migrating schistosomula and immature worms. This Th1 response is replaced at roughly six weeks post-infection, upon egg deposition, by a strong Th2 response directed at schistosome egg antigens (SEA) [23]. The Th2 immune response peaks at approximately eight weeks then is down-modulated and maintained at a decreased intensity into chronic infection. The later, egg induced, immune response has been well characterized [22, 79], but it is increasingly evident that the early immune response to schistosomes is very relevant and appears to include a diversity of Th responses [80]. The variety of host immune responses throughout schistosome infection has been demonstrated in numerous studies [78, 81-83] showing that the different life stages of the parasite contribute to the differential host immune response.

Immune responses to Schistosome Cercariae

It is well known that eggs induce a vigorous immune response that becomes down-modulated with time. Additionally, there is mounting evidence of down-regulation of the immune response as early as 48-72 hours after infection, by schistosome larvae in the skin [78]. Penetrating cercaria secrete molecules, called cercarial ES products, that activate innate antigen presenting cells (APCs)

and stimulate a variety of T cell responses, including regulatory responses, with the production of IL-12p40, IL-6, and IL-10 [84]. Schistosome larvae have also been shown to release products that stimulate dendritic cells to drive Th2 responses [85]. Cercarial ES contains the immuno-modulator Sm16 that has been shown to inhibit toll like receptor (TLR) signaling in monocytes [86]. These results indicate that, as soon as cercariae invade the skin, there are a plethora of immune responses functioning.

Immune Responses to Schistosome Worms

During pre-patent infection, schistosome worms induce a diversity of T cell responses. There exists not only a Th1 response, with the production of IFN-γ, but also a Th2 response, with $CD4^+$ T cells producing IL-4 in response to worm antigen during pre-patent infection [87]. The ES products of schistosome worms are themselves immune modulatory. Protease inhibitors derived from ES products from the helminth *Nippostrongylus brasiliensis*, have been shown to modulate innate immune function by inhibiting antigen processing, i.e. cystatin inhibits cysteine proteases that are required for antigen processing and presentation [88]. Additionally, a schistosome cysteine protease has been shown to induce an antigen-specific IgE response in an egg-independent manner [89]. Schistosome worm glycolipids are known to induce the activation of human dendritic cells, skewing them towards an inflammatory phenotype [81]. Other schistosome-expressed immunomodulatory glycoconjugates are implicated in the

modulation of macrophage [82] or dendritic cell function [90]. Specifically, schistosome lyso-phosphatidylserine (lyso-PS) stimulates dendritic cells to induce IL-10 secreting Tregs [90]. These examples detailed above further demonstrate the diverse nature of T cell responses stimulated by schistosome worm products and illustrate how schistosomes may regulate T cell responses by altering innate APC function or inducing Tregs.

Dependence of Worm Development on Host Immune Signals

Not only do schistosomes influence the host's immune response, but they also appear to co-opt host signals for their own advantage to facilitate parasite development [57, 77]. Previous studies have shown that normal worm maturation and development requires signals from the host's immune system, specifically $CD4^+$ T cells [91]. The results from this study demonstrate that worms isolated from immunodeficient recombination-activating gene $(RAG)^{-/-}$ mice, lacking T and B cells, were stunted in size as compared to worms isolated from wild type mice and did not pair or lay eggs, an important measure of sexual maturation. In infected $RAG^{-/-}$ mice, where schistosome growth and sexual maturation is impaired, adoptive transfer of $CD4^+$ T cells can restore worm development to levels comparable to that seen in infected wild type mice [92]. Furthermore, there is recent evidence that restoration of worm development does not require direct interaction between the T cell and the parasite, but rather most likely occurs via interactions with cells of the mononuclear phagocyte system

[93]. These findings highlight the potential importance of interactions between $CD4^+$ T cells and cells of the mononuclear phagocyte system (MPS), i.e. monocytes, macrophages and dendritic cells [94], in critically influencing the ability of worms to develop to sexual maturity and produce eggs, therefore propagating the infection.

Immune Responses to Schistosome Eggs

Just as T cell responses to schistosome worms are varied, so are T cell responses to schistosome eggs. The immune response to schistosome eggs has been studied extensively and is known to induce robust Th2 responses [95] coincident with the downregulation of Th1 responses [96]. Regulatory responses are also present during egg infection, where IL-10 functions to suppress T cell responses and reduce severe disease associated with schistosomiasis by interfering with egg-induced pathology [97].

As mentioned previously, most pathology in schistosomiasis is associated with an egg-induced granulomatous response that is dependent on $CD4^+$ T cells. To focus on the host's immune response in the formation of granulomas; eggs that become trapped in tissue continuously secrete SEAs and provide stimulation that leads to a vigorous immune response [23]. Balance between Th1 and Th2 responses is critical for the host during this stage of schistosomiasis, for the formation of a protective granuloma without causing excessive pathology [22]. A host protective granulomatous response is mounted to sequester toxic antigens

produced by the egg and prevent damage to host tissue. The formation of the granuloma surrounding the egg is coordinated by $CD4^+$ T cells and is composed of collagen and cells, mostly $CD4^+$ T cells, macrophages and eosinophils [98]. Although formation of the granuloma protects the host from toxic egg antigens, it is also detrimental to the host because its formation leads to fibrosis and portal hypertension. The development of fibrosis and much of the pathology is driven by Th2 type cytokines [99, 100], such as IL-13, which has been shown to be a dominant regulator of hepatic fibrosis [101]. The pathogenesis of granuloma formation requires host production of the immunoregulatory cytokine tumor necrosis factor (TNF), and interestingly, egg production by the parasite has been implicated to require TNF as well, possibly demonstrating another mechanism by which the parasite has adapted to its host [102]. Over time, the size of the granuloma and the intensity of the immune response induced by egg infection decreases; this phenomenon has been termed “endogenous desensitization” [103] and appears to be under the control of IL-12 [104].

Regulatory Response

A regulatory response exists parallel to Th2 responses induced by egg deposition [105]. Helminth infections in general are associated with the induction of an immunologically hyporesponsive state [80, 106] and are known to modulate innate or adaptive immune functions, or both [77]. The elicitation of regulatory $CD4^+$ T cells is one aspect of the hyporesponsive state where Tregs have an

important role in maintaining immune homeostasis and controlling the magnitude of the immune response to infectious disease [107]. Treg cells are a population of professional suppressor cells which function to suppress immune responses and prevent immune driven pathology that may occur due to excessive responses. They have a role during schistosome infection as specialized regulatory cells [108, 109] that aid in controlling egg-induced pathology and in down modulating the immune response to chronic egg infection. Some examples of Treg activity in schistosomiasis show that patients exhibit a hyporesponsive T cell phenotype [110, 111] and that schistosome worm derived molecules appear to induce IL-10 secreting Treg cells [90]. Treg cells may additionally influence the function of other cells, including innate immune system cells such as macrophages [112] or eosinophils [113], where both of these cell types have a role in liver granuloma formation. Previous reports also indicate a regulatory role for macrophages during schistosome infection, where a schistosome polysaccharide with immunomodulatory properties was shown to elicit a population of suppressor macrophages that suppressed $CD4^+$ T cell proliferation [82]. These suppressor macrophages may have a phenotype similar to myeloid derived suppressor cells (MDSCs), which are elicited by the tumor microenvironment and use several mechanisms to inhibit T cell proliferation [114].

It is not yet certain whether schistosome worms induce the differentiation of Treg cells or MDSC-like cells outright or whether they develop in parallel to effector T cell responses that occur. However, it does appear that Treg cells are

required for controlling liver pathology during schistosomiasis [109]. The regulatory cytokines IL-10 and TGF-β, which are characteristically produced by Tregs [115], are both important in controlling severe liver pathology during schistosomiasis [116]. The mechanism used by Tregs during schistosome infection to suppress the immune response has not been fully elucidated, but may involve the production of IL-10.

Manipulation of host immune response for therapeutic advantage

There is a need to discover new treatment methods for schistosomiasis and understanding the role of immune modulation during schistosome infection may potentially be of therapeutic value. Regulatory responses induced during schistosomiasis could be manipulated to either reduce egg-induced pathology and the formation of liver fibrosis, or ideally, help prevent or clear the infection. There is evidence from other fields of investigation that manipulation of the host's immune response could be used as a treatment for disease [117]. The manipulation of regulatory T cell responses has been explored in the fields of cancer immunotherapy and auto-immunity [118, 119]. More recently, the manipulation of T cell responses has been investigated in infectious diseases, where the goal of one particular study was to enhance T cell responses in an attempt to increase immunity to parasitic infection [120]. The identification of an essential suppressor or inhibitor protein or cytokine could lead to the development of new drugs or new vaccine strategies.

General Hypothesis

We hypothesize that $CD4^+$ T cells and innate immune cells cooperate to provide an environment that is not host protective and may be conducive to parasite establishment and development.

Specific Aims

Specific Aim 1:

We hypothesize that an immunologically hyporesponsive state is induced during pre-patent Schistosoma mansoni infection in response to schistosome worms.

Test if pre-patent *Schistosoma mansoni* infection induces an immunologically hyporesponsive state in the T lymphocyte compartment.

Sub Aim 1: Determine whether there is a regulatory T cell response present during pre-patent schistosome infection.

Sub Aim 2: Determine whether the regulatory cytokine IL-10 is produced during pre-patent schistosome infection and what role it has in modulating the immune response to schistosome worms.

Specific Aim 2:

We hypothesize that innate antigen presenting cells modulated by the presence of schistosome worms are defective in their ability to prime the adaptive CD4$^+$ T cell response to Schistosoma mansoni infection.

Investigate the role of innate antigen presenting cells in priming the adaptive CD4$^+$ T cells response to pre-patent *Schistosoma mansoni* infection.

Sub Aim 1: Examine the role of dendritic cells in the innate immune response to pre-patent *S. mansoni* infection and the functional capabilities of dendritic cells in priming the adaptive CD4$^+$ T cell immune response.

Sub Aim 2: Examine the role of mononuclear phagocytes during the innate immune response in priming the adaptive CD4$^+$ T cell response to pre-patent *S. mansoni* infection.

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Chapter 2

The Role of IL-10 and nTregs in Regulatory Responses Induced By Pre-patent Schistosome Infection

Christine E. Ferragine and Stephen J. Davies

Abstract

Schistosomes are intravascular helminths that affect approximately 200 million people throughout the world. The immune response to schistosome infection consists of an early Th1 response to developing schistosomes that is replaced at approximately 6 weeks post infection by an egg induced Th2 granulomatous response. Our data show that $CD4^+$ T cells produce IL-4, in addition to IFN-γ, in response to worm antigens during pre-patent infection. We hypothesized that the induction of regulatory responses and the production of the regulatory cytokine IL-10, creates an immunomodulatory milieu that is permissive for parasite establishment and development. Here we attempted to establish whether IL-10 is produced in response to pre-patent schistosome infection, to determine the identity of the IL-10-producing cells, and to elucidate how this response is induced. Our results show that $CD4^+$ T cells express and produce IL-10 in response to pre-patent schistosome infection. To determine whether $CD4^+CD25^+Foxp3^+$ T regulatory (Treg) cells are an important source of IL-10, wild type mice were treated with monoclonal antibodies that decrease nTreg cells, including anti-IL-2 (S4B6) and anti-CD25 (PC61) antibodies. This approach demonstrated that although the Treg population was significantly diminished, IL-10 production was not significantly reduced and suggests that another $CD4^+$ T cell population, rather than Treg cells, is the predominant source of IL-10. Further investigation is required to determine the precise identity and biological relevance of IL-10 producing $CD4^+$ T cells that are induced by schistosome worms.

Author Summary

Schistosomiasis is a disease caused by parasitic blood flukes that are found throughout the world. We have shown that the early immune response to schistosome worm infection comprises several types of T helper immune responses and that the immune regulation induced by schistosomes may be permissive for parasite establishment and development. In this study we aimed to investigate whether a regulatory response is induced my schistosome infection and to examine the cellular source of this response. Our results show that $CD4^+$ T cells both express and produce IFN-γ and IL-10 in response to schistosome infection. By inhibiting the population of natural T regulatory cells during schistosome infection we were able to assess the role of this population in producing the regulatory cytokine IL-10. It is clear that a regulatory T cell response is part of the immune response to schistosome infection, although our data indicate that natural T regulatory cells are not the predominant source of IL-10. Further investigation is required to determine the role of regulatory T cell populations during the innate immune response to schistosome infection and how signals initiating from these populations modulate the host's immune response.

Introduction

Excessive and misdirected immune responses can to catastrophic to the host. This is especially relevant in schistosomiasis, where the immune response must reach a balance between a Th2-governed granulomatous response, that functions to protect the host by sequestering toxic schistosome egg antigens, and an inflammatory Th1 response that could damage host tissue by causing severe liver pathology [1]. The immune system has evolved to develop methods to suppress excessive reactions and prevent immuno-pathological harm. In one mechanism of self tolerance, T cells control the activation and propagation of lymphocytes which are over-reactive or are responding inappropriately [2]. T cells which are specialized for controlling immune responses are called regulatory T cells (Tregs). Effector T cell and Treg cell responses have been shown to develop in parallel during the host's adaptive immune response to schistosomiasis [3]. The balance between these two T cell populations is crucial for maintaining the quality and scale of the subsequent adaptive response.

Tregs are professional suppressor cells whose function is to maintain immune homeostasis. The critical role that Tregs play in controlling excessive immune responses is evidenced in the detrimental effect of ablating the Treg population [4]. Tregs differentiate from naïve $CD4^+$ T cells and thymic precursors upon expression of the transcription factor forkhead box 3 (FOXP3) and stimulation by the cytokines transforming growth factor- β (TGF-β) and interleukin- 2 (IL-2), and characteristically produce the cytokines TGF-β and IL-10 [5]. Tregs can be broadly divided into two groups; adaptive (induced) regulatory

T cells and natural regulatory T cells. Treg cells that reside within the periphery are termed natural T regulatory (nTreg) cells [2] and typically express the IL-2 receptor alpha chain (CD25), cytotoxic T lymphocyte antigen (CTLA 4), glucocorticoid-induced tumor necrosis factor receptor family−related gene (GITR), and Foxp3 [6]. Previous reports have shown that IL-2 signaling is necessary for maintaining nTregs *in vivo* [7, 8]. NTregs are known to be associated with the response to many infectious pathogens and have been shown to be critical in limiting effector responses and damage to host tissue during the robust immune response that develops to combat infection [9].

The induction of nTregs in response to infection is critical for the host in the control of immune mediated damage, but can also to advantageous to the parasite. It is thought that immuno-regulation can facilitate parasite persistence and transmission to additional hosts [10]. In fact, the removal of nTregs during leishmania infection can lead to sterilizing immunity, which is beneficial to the host in the short term but prevents the development of long term immunity to infection [11]. Regulatory mechanisms induced during parasite infection could be therapeutically manipulated as a strategy to manage infection or enhance drug treatments and vaccination strategies.

Helminth infections in general are known to modulate innate and/or adaptive immune functions [12]. Infection with the helminth *Schistosoma mansoni* elicits the induction of a protectiveTh2-type response that down-regulates a potentially pathological Th1-type response [13], aiding in the control of egg-induced pathology. One important aspect of the development of the Th2

response during schistosomiasis includes the production of the regulatory cytokine IL-10 [14]. It has been suggested that naturally occurring Tregs are important in helminth induced immuno-modulation [9] and one mechanism that Tregs use to suppress the immune response to schistosome eggs is mediated by the production of IL-10 [15]. IL-10 independent mechanisms of suppression by Tregs have also been identified [16]. Additionally, schistosomula tegument has been shown to induce the production of the cytokine TNF-α by dendritic cells [17], where TNF-α has been implicated in the stimulation and proliferation of Tregs [18], indicating the potentially important role of Tregs in the immune response to schistosome infection.

It is known that Treg cells are important in controlling Th2 responses during chronic schistosome infection and that the production of IL-10 is critical to this response [19]. IL-10 has been implicated in the control of excessive immune responses due to both Th1 and Th2 governed mechanisms [20] and it has been found that IL-10 is capable of suppressing T cell responses in the prevention of severe pathology due to schistosome egg deposition [1]. Although the immune response to schistosome worms has been shown to include Th2 responses with the production of IL-4 [21], it has not been investigated as thoroughly as the response to schistosome eggs. However, the induction of immune responses to schistosomes is important, as the early immune response is decisive in establishing the downstream adaptive response that controls egg-induced pathology. In light of the vital role of Tregs in down-modulating the immune response to patent schistosome infection, we sought to investigate the presence

of regulatory responses induced by schistosome worms and determine the role of IL-10 during these responses to pre-patent infection. We first investigated T cell responses elicited by schistosome worms during pre-patent infection and then sought to determine whether Tregs are a critical component of the response.

Materials and Methods

Mice

C57BL/6 wild type mice were purchased from National Cancer Institute (Frederick, MD) and maintained in a pathogen free environment according to Uniformed Services University of the Health Sciences (USUHS) International Animal Care and Use Committee (IACUC) policy. All experiments using mice were performed according to USUHS IACUC policies.

S. mansoni infection

Biomphalaria glabrata snails infected with the Puerto Rican strain of *Schistosoma mansoni* were provided by Dr. Fred Lewis (BRI, Rockville, MD) and maintained in house. Mixed male and female cercariae were shed from snails by exposure to light for one hour. The concentration of cercariae was determined by counting the number present in a representative sample and mice of 4-6 weeks of age were infected percutaneously by tail immersion in water containing approximately 150 *S. mansoni* cercariae for 45 minutes [22, 23]. Mice were sacrificed at 4 weeks post infection for all experiments. Experiments included 5 mice per group with each mouse analyzed individually. Schistosome Worm Antigen Preparation (SWAP) was prepared from adult *S. mansoni* worms perfused from the portal veins of infected mice and homogenized in phosphate buffered saline on ice. Centrifugation at 16,100 g for 30 minutes at 4°C removed insoluble material and the remaining supernatant was filter sterilized and stored at -80°C, after determination of protein concentration using Bradford Assay.

Cell isolation and culture

Single cell leukocyte suspensions were obtained by forcing splenic or hepatic tissue through 70 μm nylon cell strainers. Hepatocytes were removed from liver using Percoll density gradient centrifugation per manufacturer's instructions. Briefly, 35% Percoll in RPMI 1640 (Gibco) was mixed with cell suspensions isolated from the liver. After centrifugation, the upper layer of hepatocytes was poured off, retaining the lower layer of leukocytes. Erythrocytes were lysed from hepatic and splenic leukocytes using ACK lysing buffer (Quality Biological, Inc.). $CD4^+$ T cells or $CD11c^+$ cells were isolated by magnetic cell separation after incubation with anti-CD4 or anti-CD11c microbeads, respectively, using MACS cell separation columns (Miltenyi Biotech), according to manufacturer's protocols. Magnetic isolation attained a purity of at least 95% for $CD4^+$ T cells and at least 85% for $CD11c^+$ cells and $CD11b^+$ cells. Cells from individual mice were cultured in single wells at $2x10^6$ cells/ml in RPMI 1640/10% FBS/L-glutamine (Sigma)/ Minimum Essential Medium Eagle with non-essential amino acids (MEM NEAA)(Sigma)/sodium pyruvate (Sigma)/HEPES buffer solution (Sigma)/penicillin-streptomycin (Sigma)/2-mercaptoethanol (Sigma). Antigen presenting cells were co-cultured with $CD4^+$ T cells at a ratio of 1 APC:10 $CD4^+$ T cells. Cell cultures were stimulated with SWAP at 50ug/ml [24] or anti-CD3 at 1ug/ml, clone 145-2C11 (BD Bioscience) for 72 hours at 37°C and 5% CO_2.

Quantification of cytokine production

Cells were cultured as described above and cell culture supernatants were collected after three days and stored at -80°C. Production of IFN-γ or IL-10 was measured per manufacturer's protocols using BD Opt EIA ELISA sets (BD Bioscience) and analyzed at 450 nm with λ correction 570 nm using a Spectramax M2 plate reader (Molecular Devices).

Real time PCR

RNA was extracted from the spleen or liver of wild type mice or from $CD4^+$ T cells isolated from the spleen or liver. Spleen or liver tissue was homogenized in RNAzol (Tel-tek Inc.) according to manufacturer's instructions. $10x10^6$ isolated $CD4^+$ T cells were homogenized using QIAshredder spin columns. RNA was isolated with RNeasy mini columns, with on-column DNase digestion using RNase-Free Dnase Set (Qiagen). RNA was analyzed for purity and concentration on NanoDrop ND-1000 Spectrophotometer (Wilmington, DE) and 1ug RNA used to make cDNA with High Capacity RNA to cDNA kit (Applied Biosystems) and M.J. Research DNA Engine DYAD thermal cycler (Bio-Rad). Real time PCR was performed using Taqman Gene Expression assays for interleukin-10 (IL-10), interferon-γ (IFN-γ), IL-4, tumor necrosis factor-α (TNF-α), IL-1β or Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems), per manufacturer's protocols, using M.J. Research Chromo4 PCR cycler (Bio-Rad) and Opticon Monitor 2 Analysis Software (M.J. Research) to

quantitate C_T values. Fold change was calculated using the comparative C_T method with GAPDH as the endogenous control [25].

In vivo inhibition of $CD4^+$ T cells or natural T regulatory cells

To inhibit $CD4^+$ T cells *in vivo,* mice were administered 1mg of anti-CD4 monoclonal antibody (clone GK1.5) via the intraperitoneal route [26] at 3 weeks post infection. Controls received normal Rat IgG (Sigma). At 4 weeks post infection mice were sacrificed and leukocytes from the spleen and liver were cultured as described above. Leukocytes were stained with PerCP anti-CD4 clone RM4-5 and PE anti-TCRβ (BD biosciences) and analyzed by flow cytometry. Virtually complete depletion of $CD4^+$ T cells was confirmed by flow cytometry as shown in Fig. 3.

To inhibit nTreg cells *in vivo,* 1mg of anti-CD25 (IL-2R) monoclonal antibody (clone PC61) [27] or 1mg of anti-Interleukin-2 (IL-2) monoclonal antibody (clone S4B6) [28] was administered via the intraperitoneal route at 3 weeks post infection. Control mice for these treatments received rat monoclonal IgG antibody (clone GL113) or normal Rat IgG (Sigma) respectively. Mice were sacrificed at 4 weeks post infection and splenic leukocytes were cultured as described earlier. Significant depletion of $CD4^+CD25^+Foxp3^+$ cells was confirmed by flow cytometry. Data for anti-IL-2 experiment are a combination of 4 repeat experiments.

Cellular molecule expression

For analysis of the natural regulatory T cell population from the spleen of wild type mice, freshly isolated cells were stained following manufacturer's protocol using the mouse regulatory T cell staining kit (eBioscience) and staining cells with fluorescein isothiocyanate-conjugated anti-CD4 clone RM4-5, allophycocyanin-conjugated anti-CD25 clone PC61 or 3C7, and phycoerythrin-conjugated anti-FOXP3 clone FJK-16s. Cells were gated on forward scatter (FSC-H/FSC-A) to exclude doublets and on FSC-H and side scatter (SSC-H) to exclude granulocytes. The population of nTreg cells was analyzed by first gating on the $CD4^+$ population, then the $CD25^+$ and $Foxp3^+$ cell population, as shown in Fig. 4. All samples were analyzed on a LSR II Optical Bench Flow Cytometer using FACSDiva (BD Biosciences) and Winlist software (Verity Software House).

Statistical analysis

Due to unequal variances among some of the experimental groups analyzed, non parametric tests were used to test for significant differences between groups. For comparisons between two groups, Mann-Whitney test was used and for comparisons between more than two groups, Kruskal-Wallis test was used followed by Dunn's multiple comparison tests. GraphPad Prism Software Version 5 (GraphPad Software Inc., San Diego, CA) was used to perform all statistical analyses. *P* values of 0.05 or less were considered significant. All experiments were repeated at least twice with 5 animals per group.

Figure 3. Depletion of $CD4^+$ T cells

Wild type mice were administered 1mg of anti-CD4 monoclonal antibody clone GK1.5 (Fig. 3B, D) or control Rat IgG (Fig. 3A, C) via the intraperitoneal route at 3 weeks post schistosome infection. At 4 weeks post infection, mice were sacrificed and leukocytes from the spleen (Fig. 3A, B) and liver (Fig. 3C, D) were isolated. Cells were stained with PerCP anti-CD4 clone RM4-5 and PE anti-TCRβ (BD biosciences) and analyzed by flow cytometry. αCD4, anti-CD4 monoclonal antibody; control, control Rat IgG monoclonal antibody.

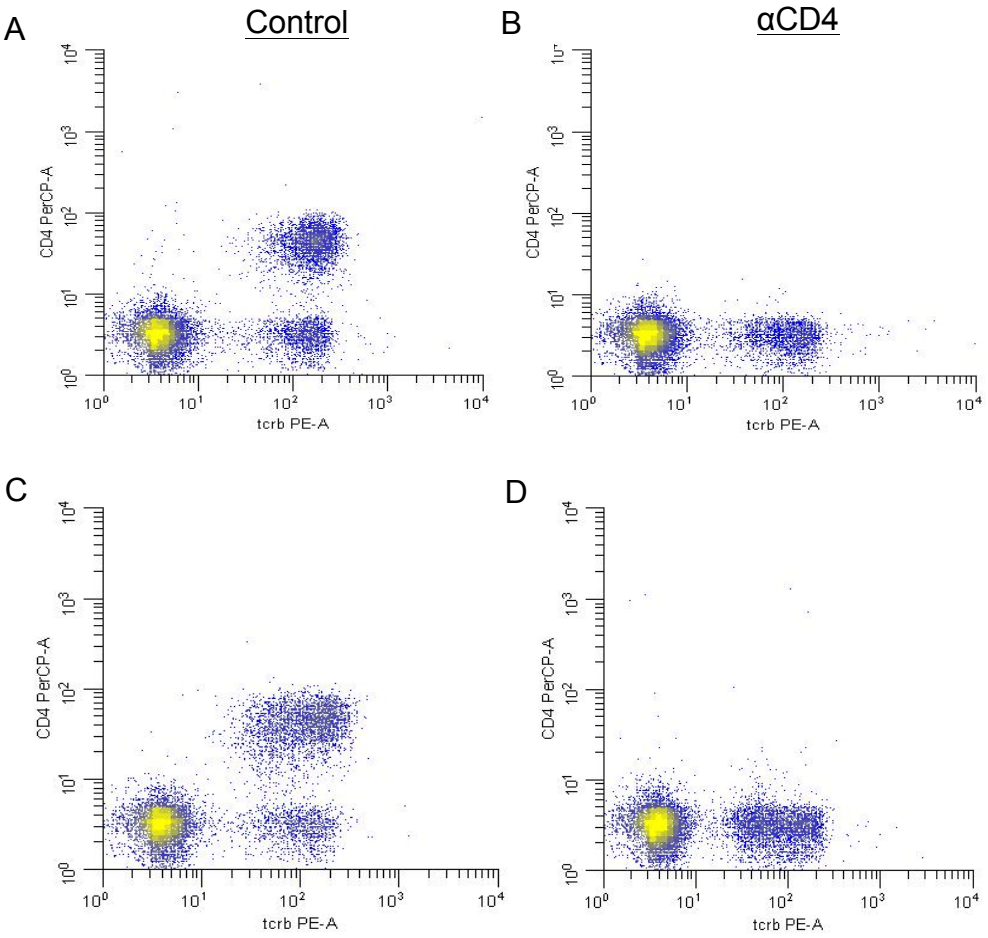
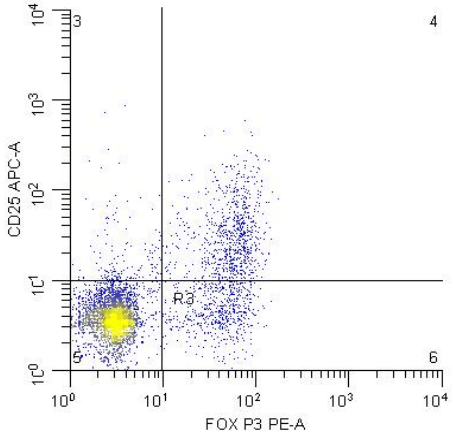

A
Control
B
αCD4
C
D
CD4 PerCP-A
tcrb PE-A
10^0
10^1
10^2
10^3
10^4

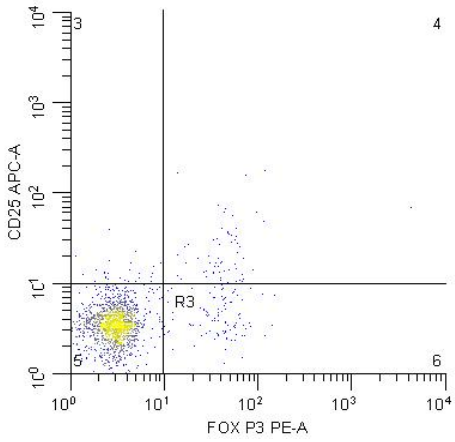
Figure 4. nTreg population in the spleen and liver

Leukocytes freshly isolated from the spleen and liver of non-infected wild type mice were gated on forward scatter (FSC-H/FSC-A) to exclude doublets and on FSC-H and side scatter (SSC-H) to exclude granulocytes. The population of nTreg cells was analyzed by first gating on the $CD4^+$ population, then the $CD25^+$ and $Foxp3^+$ cell population.

Spleen



Liver



Results

Pre-patent schistosome infection induces the expression IL-10 as well as IFN-γ and IL-4 by $CD4^+$ T cells

To examine the role of immuno-regulation during pre-patent schistosome infection, we investigated the expression of the regulatory cytokine IL-10 as well as IFN-γ and IL-4. RT-PCR performed on RNA extracted from $CD4^+$ T cells freshly isolated from 4 week-infected wild type mice showed that splenic $CD4^+$ T cells significantly up-regulate expression of IFN-γ (Fig. 5A), IL-4 (Fig. 5B) and IL-10 (Fig. 5C) in response to pre-patent infection.

$CD4^+$ T cells produce IFN-γ and IL-10 in response to pre-patent infection

To determine whether splenic $CD4^+$ T cells contribute to the production of IL-10, $CD4^+$ T cells were ablated in infected wild type mice using an anti-CD4 monoclonal antibody. Control infected mice were treated with a normal Rat IgG antibody. Virtually complete depletion of $CD4^+$ cells in the anti-CD4 treated animals was confirmed by flow cytometry (Fig. 3). *In vitro* recall response assays performed on splenocytes showed that $CD4^+$ T cell depletion led to a significant inhibition of IFN-γ (Fig. 6A) and IL-10 (Fig. 6B) production in response to SWAP stimulation as compared to control treated mice. There remained a small production of IL-10 by splenocytes from mice depleted of $CD4^+$ T cells; this IL-10 is likely produced by cells other than $CD4^+$ T cells, such as B cells or myeloid cells. There was also a reduction in the production of IFN-γ in response to SWAP by cells from the liver of mice depleted of $CD4^+$ T cells (Fig. 6C). The

overall level of cytokine production by cells from the liver is much less than that seen with splenocytes. However, the SWAP stimulated production of IL-10 by cells from the liver of infected mice depleted of $CD4^+$ T cells was also significantly less than in control treated mice (Fig. 6D). These data indicate that $CD4^+$ T cells are the main cellular source of both IFN-γ and IL-10 during pre-patent infection.

In vivo inhibition of IL-2 signaling during pre-patent schistosome infection does not significantly reduce IFN-γ or IL-10 production

To test whether the $CD4^+$ T cells producing IL-10 are nTregs, and if nTregs are an important source of IL-10 during pre-patent infection, nTregs were reduced *in vivo* by inhibiting IL-2 signaling. It has been shown that IL-2 signaling is required to maintain nTregs *in vivo* [7, 8], and blocking IL-2 signaling will lead to a reduction in the nTreg population [7]. An anti-IL-2 monoclonal antibody was used to decrease the population of $CD4^+$ T cells that are $CD25^+Foxp3^+$ in infected wild type mice. A group of infected control mice were treated with normal Rat IgG. The population of nTregs was significantly reduced in anti-IL-2 treated mice compared to control treated mice (Fig. 7A). $CD4^+$ T cells isolated from the spleen of anti-IL-2 treated and control treated mice were co-cultured with $CD11c^+$ dendritic cells isolated from the spleen of non-infected mice for three days without antigen or in the presence of SWAP. Cytokine concentrations in the cell culture supernatant were measured by ELISA. While Anti-IL-2 antibody treatment significantly reduced the nTreg population, the production of IFN-γ (Fig. 7B) and IL-10 (Fig. 7C) by T cells from infected mice treated with anti-IL-2

antibody in response to SWAP was not significantly reduced when compared to T cells from infected mice treated with control antibody. These data suggest that nTregs are likely not the predominant cellular source of IL-10 and are also not likely to be important in controlling the IFN-γ response induced by pre-patent schistosome infection.

Significantly reducing population of $CD25^+$ nTregs during pre-patent schistosome infection does not alter production of IFN-γ or IL-10

To further test whether the $CD4^+$ T cells producing IL-10 are nTregs, the population of $CD4^+CD25^+Foxp3^+$ nTreg cells was depleted in infected mice using an anti-CD25 (anti-IL-2R) monoclonal antibody. The anti-CD25 antibody treatment significantly reduced the population of nTregs in the spleens of infected mice when compared to control infected mice (Fig. 8A). Splenic $CD4^+$ T cells were isolated from anti-CD25 treated and control treated mice and co-cultured with splenic $CD11c^+$ dendritic cells isolated from non-infected mice for three days without antigen or in the presence of SWAP. Cell culture supernatants were analyzed for cytokine secretion. In comparison to T cells from control mice, The production of IFN-γ and IL-10 by T cells from infected mice treated with anti-CD25 in response to SWAP was not significantly altered (Fig. 8B, Fig. 8C). Although there is a slight decrease in the production of SWAP stimulated IL-10 by cells from anti-CD25 treated mice (Fig. 8C), there is no coordinating increase in IFN-γ production when cells from anti-CD25 treated mice are stimulated with SWAP (Fig. 8B). This may be a result of a deficiency in the methodology of reducing $CD25^+$ cells, as we may also be targeting activated T cells which would

function in producing IFN-γ. We conclude that although nTregs do produce IL-10 during schistosome infection [15], they are not significant contributors during pre-patent infection to the production of this cytokine by $CD4^+$ T cells as a whole. Furthermore, our data suggest that nTreg cells may not be important in controlling IFN-γ production by CD4 T cells in response to pre-patent schistosome infection.

Discussion

The innate immune response to schistosome worms is critical in structuring the subsequent development of the adaptive response [12, 29]. This sequence of events is made even more important by the fact that it is the adaptive response which controls damage to the host due to egg induced pathology. It is known that the adaptive response to schistosome eggs becomes down modulated with time and continues at a decreased intensity during chronic schistosome infection [29]. This immunomodulation has been shown to occur not only in response to schistosome eggs, but has been described much earlier during schistosome infection, where migrating schistosomula in the skin stimulate cells of the innate immune system [30]. Schistosomulae have also been shown to induce immuno-regulation by secreting an anti-inflammatory protein [31] or suppressing T cell proliferation in an IL-10 dependant manner by inducing the production of the eicosanoid PGE_2 [32]. Thus investigating this early regulation of the immune response may identify new targets for drug therapies or vaccination strategies.

The pre-patent immune response to schistosome infection is characterized by diverse T cell responses, including Th1 [29], Th2 [21] and regulatory responses [32]. We specifically investigated the $CD4^+$ T cell response by measuring the expression of IFN-γ, IL-4, and IL-10 by isolated $CD4^+$ T cells and found these cytokines were all up-regulated by pre-patent schistosome infection. Our data also show that TNF-α is up-regulated in the liver during infection and remains stable in the spleen (Fig. 9A). This result correlates with

the finding that TNF-α stimulates the activation and proliferation of Tregs, resulting in the up-regulation of FOXP3 on Tregs and increasing their suppressive ability [18]. This finding is also specifically relevant to our investigation of regulatory responses during pre-patent schistosome infection in light of the fact that schistosomula tegument has been shown to cause dendritic cells to produce TNF-α [17]. The pro-inflammatory molecule, IL-1β, is also produced by a variety of cells. Schistosome egg antigen (SEA) has been implicated in the activation of the Nlrp3 inflammasome and induction of IL-1β production [33]. However, schistosome worms do not seem to induce the production of IL-1β during pre-patent infection, where the expression of this cytokine was found to be down-regulated in the spleen during infection (Fig. 9B). Taken together, these data suggest that the cytokine response to pre-patent schistosome infection is both diverse and complicated.

We focused on the regulatory role of IL-10 because the production of IL-10 by regulatory $CD4^+$ T cells is known to be an important aspect of the immuno-modulation that occurs during the adaptive response to schistosome eggs [15]. We have previously established that schistosome worms induce the production of IL-4 and IFN-γ [21]. In this study we aimed to investigate the role of $CD4^+$ T cells in the regulatory response that appears to be induced by schistosome worms. By depleting the population of $CD4^+$ T cells *in vivo* during pre-patent schistosome infection we were able to assess their contribution to the production of IFN-γ and IL-10. Schistosome infected mice depleted of $CD4^+$ T cells showed a significant reduction in IFN-γ and IL-10 production, suggesting that $CD4^+$ T cells

are the main producers of IL-10 during pre-patent schistosome infection. The remaining production of IL-10 may come from $CD8^+$ T cells [34], although there is a lack of evidence to suggest that $CD8^+$ T cells produce IL-10 in response to schistosome infection, or may also be produced by B cells or myeloid cells. While our methodology did result in a significant reduction in IL-10 production by depleting mice of $CD4^+$ T cells, the use of flow cytometric analyses and intracellular cytokine staining would be a more direct way to assess which cell populations are responsible for the production of IL-10 in response to schistosome infection.

We next wanted to examine the role of natural T regulatory cells in the production of IFN-γ and IL-10 by $CD4^+$ T cells, as it is thought that nTregs are important in the immuno-modulation that is mediated by helminth infection [9] . Two different methods were used to reduce the population of $CD4^+CD25^+FOXP3^+$ nTregs *in vivo*; blocking IL-2 signaling, which is critical for the function and maintenance of nTregs [7], or depleting the population of $CD25^+$ cells. Both of these methods significantly reduced the population of nTregs in the spleen of treated mice as compared to control treated mice. However, in neither case was splenocyte production of IFN-γ or IL-10 significantly altered by reduction of the nTreg population. Our data may suggest that nTregs are not the predominant source of IFN-γ or IL-10 during pre-patent schistosome infection. The limitation to our nTreg depletion method is that the population was not ablated, although the significant reduction of the nTreg population should alter cytokine levels if nTregs are in fact significant producers of IFN-γ or IL-10.

Alternate methods could include the use of mice deficient in genes essential for nTreg development or maintenance. Additionally, our lab has identified IL-10 as being critical in the control of Th1 responses, with the production of IFN-γ, during schistosome infection, where infected IL-10 knock-out mice demonstrated unrestrained production of IFN-γ in response to worm antigen (Mazen Makarem, unpublished data). This finding in conjunction with the data presented may indicate that nTregs are not critical in controlling the production of IFN-γ during schistosome infection.

Our data indicate that $CD4^+$ T cells express IL-10 during pre-patent schistosome infection and produce IL-10 in response to schistosome worm antigen. The specific sub-population of $CD4^+$ T cells that are vital in mediating the regulation governed by IL-10 during infection has yet to be identified. Our data indicate that nTregs are not the predominant population of cells responsible for producing IL-10 in response to pre-patent schistosome infection and may also not be essential in controlling the production of IFN-γ. IL-10 could be produced by an inducible population of $CD4^+$ T cells such as Tr1 cells, which have been shown to differentiate from immature populations of T cells dependent on their environment [2]. Tr1 cells are capable of producing IL-10 and TGF-β and lack FOXP3 expression [35]. However, further work is required to determine which cellular populations mediate the regulatory response to schistosome worms and whether this response is governed by IL-10 or another immuno-suppressive cytokine.

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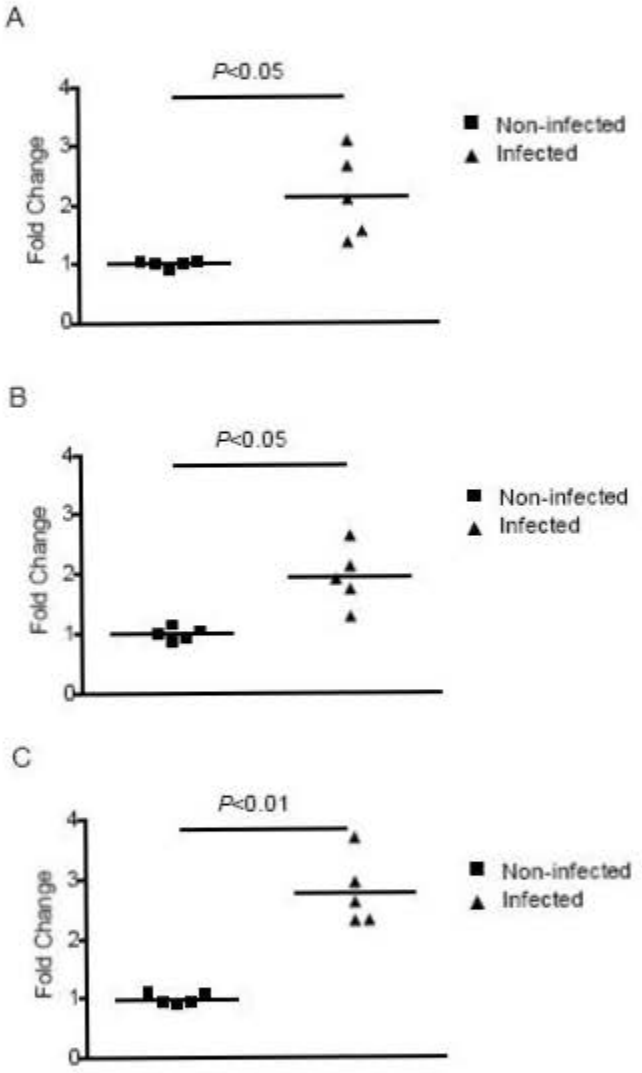
Figure 5. Pre-patent schistosome infection induces the expression IL-10 as well as IFN-γ and IL-4 by $CD4^+$ T cells

$CD4^+$ T cells were magnetically isolated from the spleens of four week infected or non-infected wild type mice. RNA was extracted from $CD4^+$ T cells and cDNA synthesized. Real time PCR was performed using Taqman Gene Expression assays for IFN-γ (Fig. 3A), IL-4 (Fig. 3B) or IL-10 (Fig. 3C), and fold change was calculated using the comparative C_T method with GAPDH as the endogenous control. Horizontal bars indicate the mean of five individual mice.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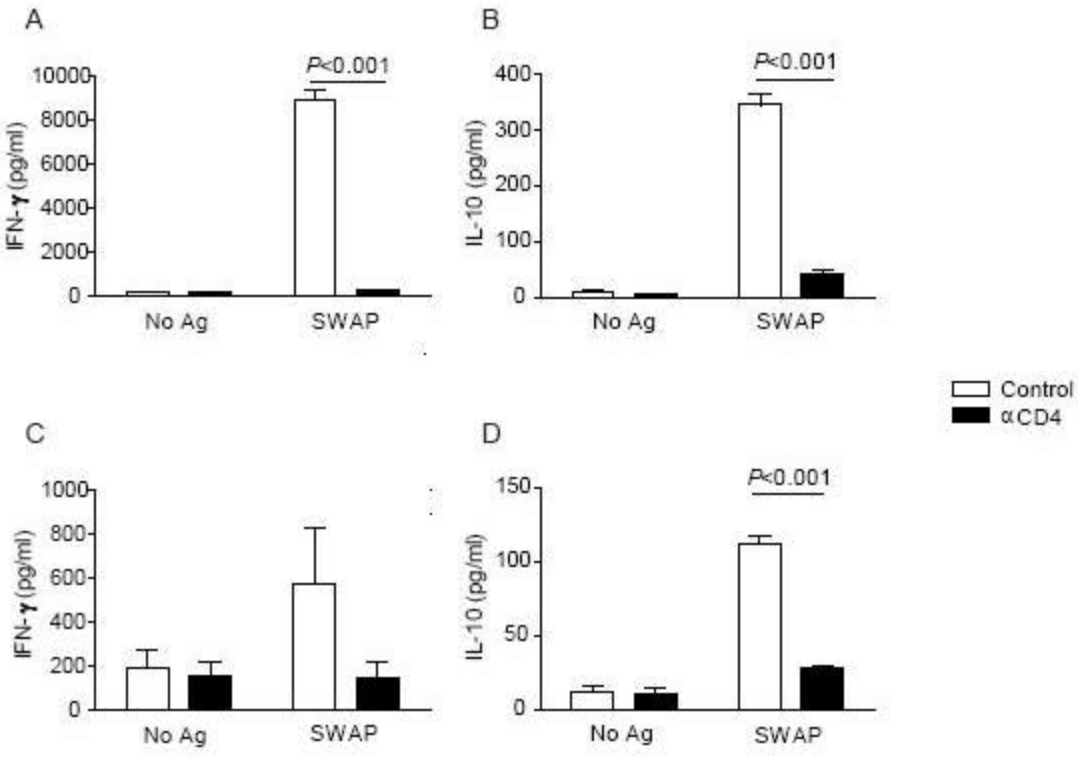
P<0.05
Fold Change
0
1
2
3
4
Non-infected
Infected
B
P<0.05
Fold Change
0
1
2
3
4
Non-infected
Infected
C
P<0.01
Fold Change
0
1
2
3
4
Non-infected
Infected

Figure 6. $CD4^+$ T cells produce IFN-γ and IL-10 in response to pre-patent infection

Wild type mice at three weeks post *S. mansoni* infection were injected intra-peritoneal with 1mg of anti-CD4 monoclonal antibody, clone GK1.5, or control Rat IgG antibody. At four weeks post infection, leukocytes were isolated from the spleen (Fig. 4A, B) or liver (Fig. C, D) and cultured for three days without antigen or with SWAP stimulation. Flow cytometric analysis was used to confirm ablation of $CD4^+$ T cells. Cell culture supernatant was analyzed by ELISA for production of IFN-γ (Fig. 4A, C) or IL-10 (Fig. 4B, D). Data is represented as mean +/- SEM. Data are representative of three repeat experiments. No Ag, no antigen; SWAP, soluble worm antigen preparation; αCD4, anti-CD4 monoclonal antibody.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B
C
D
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P<0.001
P<0.001
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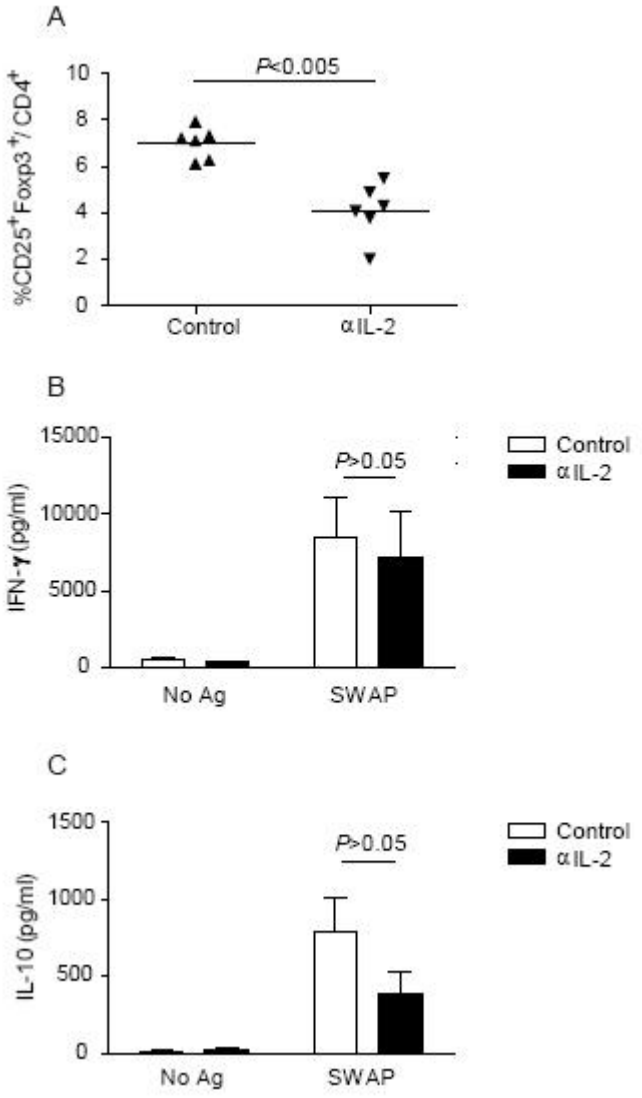
IL-10 (pg/ml)
IFN-γ (pg/ml)
IL-10 (pg/ml)
10000
8000
6000
4000
2000
0
400
300
200
100
0
1000
800
600
400
200
0
150
100
50
0
No Ag
SWAP
No Ag
SWAP
No Ag
SWAP
No Ag
SWAP
Control
αCD4

Figure 7. In vivo inhibition of IL-2 signaling during pre-patent schistosome infection does not significantly reduce IFN-γ or IL-10 production

Wild type mice were injected intra-peritoneal with 1mg of anti-Interleukin-2 (IL-2) monoclonal antibody, clone S4B6, or control Rat IgG antibody at three weeks post *S. mansoni* infection. At four weeks post infection, splenic leukocytes were isolated and flow cytometric analysis was used to examine the population of $CD4^+CD25^+FOXP3^+$ cells from anti-IL-2 and control treated mice (Fig. 5A). Horizontal bars indicate the mean of five individual mice. $CD4^+$ T cells were isolated from the spleen of anti-IL-2 and control treated mice and co-cultured with $CD11c^+$ dendritic cells isolated from non-infected mice for three days without antigen or with SWAP stimulation. Cell culture supernatant was analyzed by ELISA for production of IFN-γ (Fig. 5B) or IL-10 (Fig. 5C). Data is represented as mean +/- SEM. Data represent four repeat experiments. No Ag, no antigen; SWAP, soluble worm antigen preparation; αIL-2, anti-IL-2 monoclonal antibody.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6
4
2
0
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αIL-2
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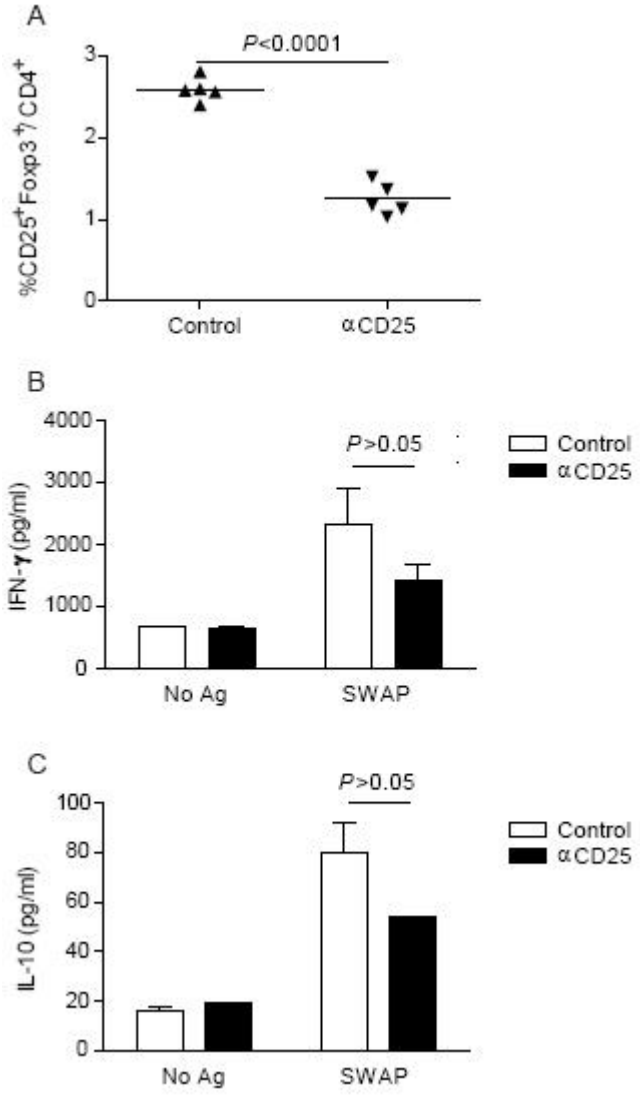
IFN-γ (pg/ml)
15000
10000
5000
0
No Ag
SWAP
Control
αIL-2
C
P>0.05
IL-10 (pg/ml)
1500
1000
500
0
No Ag
SWAP
Control
αIL-2

Figure 8. Significantly reducing population of $CD25^+$ nTregs during pre-patent schistosome infection does not alter production of IFN-γ or IL-10

Wild type mice were injected intra-peritoneal with 1mg of anti-CD25 (IL-2R) monoclonal antibody, clone PC61, or control Rat IgG antibody at three weeks post *S. mansoni* infection. At four weeks post infection, splenocytes were isolated and flow cytometric analysis was used to examine the population of $CD4^+CD25^+FOXP3^+$ cells from anti-IL-2R and control treated mice (Fig. 6A). Horizontal bars indicate the mean of five individual mice. $CD4^+$ T cells were isolated from the spleen of anti-IL-2R and control treated mice and co-cultured with $CD11c^+$ dendritic cells isolated from non-infected mice for three days without antigen or with SWAP stimulation. ELISA was used to analyze cell culture supernatant for production of IFN-γ (Fig. 6B) or IL-10 (Fig. 6C). Data is represented as mean +/- SEM. Data are representative of three repeat experiments. No Ag, no antigen; SWAP, soluble worm antigen preparation; αCD-25, anti-CD25 monoclonal antibody.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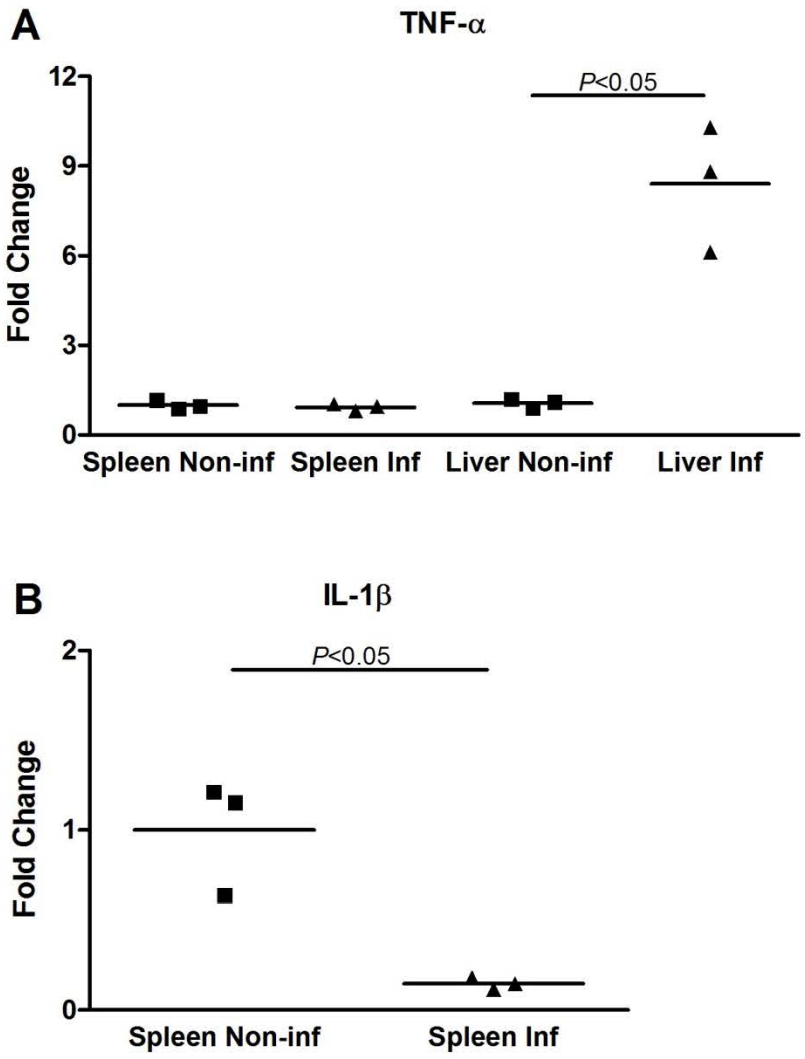
%CD25+Foxp3+/ CD4+
3
2
1
0
Control
αCD25
B
P>0.05
IFN-γ (pg/ml)
4000
3000
2000
1000
0
No Ag
SWAP
Control
αCD25
C
P>0.05
IL-10 (pg/ml)
100
80
60
40
20
0
No Ag
SWAP
Control
αCD25

Figure 9. Expression of TNF-α and IL-1β in response to pre-patent schistosome infection

RNA was extracted from spleen or liver tissue of four week infected or non-infected wild type mice. cDNA was synthesized and real time PCR performed using Taqman Gene Expression assays for TNF-α and IL-1β. Fold change was calculated using the comparative C_T method with GAPDH as the endogenous control. Horizontal bars indicate the mean of three individual mice.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TNF-α
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Fold Change
12
9
6
3
0
Spleen Non-inf
Spleen Inf
Liver Non-inf
Liver Inf
B
IL-1β
P<0.05
Fold Change
2
1
0
Spleen Non-inf
Spleen Inf

Chapter 3

Inhibition of $CD4^+$ T Cell Responses by Impairment of Myeloid Antigen-Presenting Cell Function During Pre-patent Schistosome Infection

Christine E. Ferragine and Stephen J. Davies

Manuscript submitted for publication review to PLoS Neglected Tropical Diseases

Abstract

Schistosomes are intravascular helminths that infect over 200 million people worldwide. Accumulation of schistosome eggs in host tissues stimulates Th2 responses to egg antigens and induces granulomatous pathology that is a hallmark of schistosome infection. However, prior to oviposition, the migration and development of juvenile schistosomes in the vasculature also induces immune responses to worm antigens, which may influence host resistance and susceptibility to schistosomes and concomitant infections. Here we provide evidence that the host response to pre-patent schistosome infection is associated with a loss of T cell responsiveness to other antigens. Our data suggest this loss of T cell responsiveness is due to a specific diminution in the ability of innate antigen-presenting cells to stimulate T cells. Diminution of stimulatory capacity by schistosome infection specifically affected $CD11b^+$ cells. We did not find evidence for production of a diffusible inhibitor by innate cells from infected mice. Rather, inhibition of T cell stimulation by cells from infected mice required cell contact and only occurred when suppressive cells outnumbered competent APCs by 3:1. Finally, we provide evidence that loss of T cell stimulatory capacity may in part be due to suppression of IL-12 production by pre-patent schistosome infection. Modulation of $CD4^+$ T cell and mononuclear phagocyte function during pre-patent schistosome infection is an intriguing finding, as we recently showed that both these cell populations may be exploited by schistosomes to facilitate their normal development.

Author Summary

The disease schistosomiasis is caused by a parasitic blood fluke found mainly in the tropics and subtropics and affects over 200 million people worldwide. Using mice to model human schistosome infection, our previous studies showed that schistosome development in the infected host is linked to host immune function, such that parasite development is impaired in hosts with immunological deficiencies. $CD4^+$ T cells and cells of the monocyte/macrophage lineage are two types of immune cells that are involved in modulating schistosome development. In this study, we examined immune function in mice infected with developing schistosomes, to gain insights into how immune cells might influence parasite development. We found evidence of broad-spectrum suppression of $CD4^+$ T cell responses during early schistosome infection. We also show that the loss of T cell responsiveness is due to impairment of T cell stimulation by $CD11b^+$ cells. These findings suggest that exploitation of $CD4^+$ T cells and monocytes/macrophages by schistosomes may involve parasite manipulation of the function of these cells.

Introduction

Schistosomes are intravascular helminths affecting approximately 200 million people throughout the tropics and subtropics [1, 2], with more than 90% of cases occurring in sub-Saharan Africa [3]. Upon infection, a variety of host responses are induced. Exposure of antigen-presenting cells (APCs) in the skin to invading cercariae stimulates their migration to the draining lymph nodes and induction of transient parasite-specific T helper (Th) 2 responses [4]. While mononuclear cells and neutrophils infiltrate the skin in response to the penetration of cercariae [5], evidence suggests that schistosomula in the skin elicit an immuno-modulatory environment, by secreting an anti-inflammatory protein [6] and inducing the production of the eicosanoid, prostaglandin E-2 (PGE_2), which suppresses T cell proliferation in an interleukin (IL-) 10-dependent manner [7]. Onward parasite migration into the circulatory system results in a mixed response, with evidence of both Th2 [8] and modest Th1 induction [9]. The former is necessary and sufficient to induce production of antigen-specific IgE and sensitization of basophils to produce further IL-4 in response to worm antigens [8]. At roughly 5-6 weeks post infection, parasite egg production commences and stimulates a robust, predominantly Th2 response [10] [11], while prior responses to worm antigens are down-regulated [9].

Schistosomes can persist in the host for an average of 5-10 years [12] evading immune destruction to establish long term, chronic infections [1]. Chronic infections in general [13, 14] and helminth infections in particular [15, 16] are associated with the induction of an immunologically hyporesponsive state

where either innate or adaptive immune functions, or both, are modulated [17-19]. Examples of modulation in innate immune function by helminths have been documented in the literature. For instance, protease inhibitors found in helminth excretory-secretory (ES) products, such as cystatins, inhibit cysteine proteases required for APC antigen processing and presentation [20]. Helminth cystatins also elicit the production of the immunosuppressive cytokine IL-10, reducing expression of co-stimulatory molecules on APCs and inhibiting T cell proliferation [21]. Other secreted helminth products, such as ES-62 and schistosome-expressed glycoconjugates, suppress macrophage IL-12 production [22] and induce suppressor macrophages [23], respectively. Finally, schistosome lyso-phosphatidylserine (lyso-PS), another immunomodulatory glycoconjugate, stimulates dendritic cells (DC) to induce IL-10 secreting Tregs, leading to regulation of the T cell response [24].

Modulation of the antigen-presenting capabilities of innate cells by helminths may subsequently be implicated in the modulation of adaptive immune function and the induction of regulatory T (Treg) cell responses later in infection [25]. The regulatory cytokine IL-10 has been shown to inhibit T cell activation by downregulating MHCII and B7 expression on APCs [26], thus leading to decreased T cell responses. In the case of schistosomes, patients chronically infected with *S. haematobium* [27, 28] and with *S. mansoni [29]* exhibit hyporesponsiveness in the T lymphocyte compartment, characterized by reduced proliferation and interferon (IFN-) γ production in response to parasite antigen. Not only is the parasite-specific T cell response down modulated [30], but so is

the overall T cell response that is elicited by polyclonal stimulation [31]. There is speculation that the immune modulation brought about by helminth infection may in fact be an adaptive mechanism used by worms for their own advantage, as preventing destructive immunopathology prolongs survival of both parasite and host [17, 32].

Previous data suggest the immunoregulatory state may have implications for the subsequent pathology induced by schistosome eggs [33-35]. There is a careful balance between Th1 and Th2 responses which must be maintained in order to decrease immuno-pathological damage to the host. Egg production during *S. mansoni* infection is associated with disease and leads to a strong Th2 response [11], coincident with the down-regulation of a potentially protective Th1 response [9]. Excessive Th1 [36] or Th2 responses are distinct, but both are detrimental to the host and can be suppressed by IL-10 [35]. IL-10 mediated suppression of T helper responses has been shown to prevent pathogenesis associated with schistosomiasis by interfering with the development of severe egg-induced pathology [37].

There is a need for new treatment methods for schistosomiasis as there currently exists only one effective drug, praziquantel, and schistosome strains tolerant to praziquantel have been found in endemic areas [38]. Understanding how schistosome worms influence the host's cellular immune response could afford the ability to therapeutically manipulate the immune response in an effort to protect the host, by either reducing egg-induced pathology or, ideally, by clearing or preventing infection. Manipulation of Treg responses to alter the

course of disease has been explored in the fields of cancer immunotherapy, auto-immunity [39, 40], and infectious diseases. In a model of *Leishmania* infection, Treg cells were responsible for the persistence of small numbers of parasites at the sight of infection, whereas inhibition of Treg responses allowed for sterilizing immunity [41], illustrating the potential usefulness of Treg manipulation in the treatment of parasitic infections.

Using mouse models of *Schistosoma mansoni* infection, we have previously shown that normal worm maturation and development requires host immune signals, specifically $CD4^+$ T cells [42]. In immunodeficient $RAG^{-/-}$ mice, lacking T and B cells, schistosome growth and sexual maturation is impaired, and adoptive transfer of $CD4^+$ T cells can restore worm development [43]. It is clear that $CD4^+$ T cells are critical for schistosome worm development, yet the exact mechanism for this dependence is not certain. There are solid implications that schistosome worms could be inducing a regulatory response during pre-patent infection. As alluded to earlier, the parasite's dependence on the host's immune system and the potential induction of a regulatory response may be a consequence of the co-evolution of the host and parasite and may prove advantageous for parasite persistence. In this study we looked at T cell responses to parasite antigen during pre-patent schistosome infection and aimed to investigate whether immunomodulation is induced by schistosome worms. We have observed a global impairment of T cell responses to non-parasite antigen. This impairment is governed by a defect in the myeloid cell population, where they are incapable of functioning as antigen presenting cells.

Materials and Methods

Mice

C57BL/6 wild type mice were purchased from National Cancer Institute (Frederick, MD) and maintained in a pathogen free environment according to Uniformed Services University of the Health Sciences (USUHS) International Animal Care and Use Committee (IACUC) policy. RAG-1$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in house to attain the required number of animals for experiments. OT-II mice [44] were bred in house with RAG-1$^{-/-}$ mice to attain OT-II/RAG-1$^{-/-}$ mice. All experiments using mice were performed according to USUHS IACUC policies.

S. mansoni infection

Infected *Biomphalaria glabrata* snails were provided by Dr. Fred Lewis (BRI, Rockville, MD) and maintained in house, mixed male and female cercariae of the Puerto Rican strain of *Schistosoma mansoni* were shed from snails. The concentration of cercariae was determined by counting the number present in a representative sample and mice of 4-6 weeks of age were infected percutaneously by tail immersion in water containing approximately 150 *S. mansoni* cercariae for 45 minutes [45, 46]. For all experiments mice were sacrificed at 4 weeks post infection and included 5 mice per group with each mouse analyzed individually. Schistosome Worm Antigen Preparation (SWAP) was prepared from adult *S. mansoni* worms perfused from the portal veins of infected mice and homogenized in phosphate buffered saline on ice.

Centrifugation at 16,100 g for 30 minutes at 4°C removed insoluble material and the remaining supernatant was filter sterilized and stored at -80°C after determination of protein concentration using Bradford Assay.

Cell isolation and culture

Single cell leukocyte suspensions were acquired by forcing splenic, hepatic or lymph node tissue through 70 µm nylon cell strainers. Hepatocytes were removed from liver using Percoll density gradient centrifugation per manufacturer's instructions. Briefly, 35% Percoll in RPMI 1640 (Gibco) was mixed with cell suspensions isolated from the liver. After centrifugation the upper layer of hepatocytes was poured off, retaining the lower layer of leukocytes. Erythrocytes were lysed from hepatic and splenic leukocytes using ACK lysing buffer (Quality Biological, Inc.). $CD4^+$ T cells, $CD11c^+$, or $CD11b^+$ cells were isolated by magnetic cell separation after incubation with anti-CD4, anti-CD11c, or anti-CD11b microbeads, respectively, using MACS cell separation columns (Miltenyi Biotech) according to manufacturer's protocols. Magnetic isolation attained a purity of at least 95% for $CD4^+$ T cells and at least 85% for $CD11c^+$ cells and $CD11b^+$ cells. Cells from individual mice were cultured in single wells at $2x10^6$ cells/ml in RPMI 1640/10% FBS/L-glutamine (Sigma)/ Minimum Essential Medium Eagle with non-essential amino acids (MEM NEAA)(Sigma)/sodium pyruvate (Sigma)/HEPES buffer solution (Sigma)/penicillin-streptomycin (Sigma)/2-mercaptoethanol (Sigma). Antigen presenting cells were co-cultured with $CD4^+$ T cells at a ratio of 1 APC:10 $CD4^+$ T cells. Cell cultures were stimulated with SWAP at 50ug/ml [47], $OVA_{323-339}$ peptide at 2.5ug/ml

(Anaspec), anti-CD3 at 1ug/ml, clone 145-2C11 (BD Bioscience), IL-2 at 5ul/ml, or rIL-12 at 100ng/ml (Peprotech) for 72 hours at 37°C and 5% CO_2. Cell cultures were inhibited using N^{ω}-hydroxy-nor-Arginine (nor-NOHA) at 500ug/ml (Calbiochem), L-N^{G}-monomethyl Arginine citrate (L-NMMA) at 500 ug/ml (Calbiochem), anti-IL-10R monoclonal antibody at 10ug/ml (1B1.3a hybridoma kindly provided by Dr. Yasmine Belkaid), anti-CD274 (PD-L1) monoclonal antibody at 500ng/ml, clone MIH5 (eBioscience), or anti-TGF-β1, 2, 3 monoclonal antibody at 1.5ug/ml, clone 1D11 (R&D Systems).

Quantification of cytokine production

Cells were cultured as described above and supernatants were collected and stored at -80°C. Production of IFN-γ, IL-10, or IL-4 was measured per manufacturer's protocols using BD Opt EIA ELISA sets (BD Bioscience) and analyzed at 450 nm with λ correction 570 nm using a Spectramax M2 plate reader (Molecular Devices).

Real time PCR

Splenic or hepatic tissue from four week *S.mansoni*-infected or non-infected wild type mice was homogenized in RNAzol (Tel-tek Inc.) per manufacturer's instructions and RNA was isolated with RNeasy mini columns, with on-column DNase digestion using RNase-Free Dnase Set (Qiagen). RNA was analyzed for purity and concentration was quantitated on NanoDrop ND-1000 Spectrophotometer (Wilmington, DE) and 1ug RNA used to make cDNA with High Capacity RNA to cDNA kit (Applied Biosystems) and M.J. Research

DNA Engine DYAD thermal cycler (Bio-Rad). Real time PCR was performed using Taqman Gene Expression assays for IL-12p35 and GAPDH (Applied Biosystems), per manufacturer's protocols, using M.J. Research Chromo4 PCR cycler (Bio-Rad) and Opticon Monitor 2 Analysis Software (M.J. Research) to quantitate C_T values. Fold change was calculated using the comparative C_T method with GAPDH as the endogenous control[48].

Cell surface molecule expression

For analysis of APC populations, cells isolated from spleen and liver of wild type mice were stained with Live-Dead Aqua fixable dead cell stain (Invitrogen), phycoerythrin-conjugated anti-B220 clone RA3-6B2, allophycocyanin-conjugated anti-CD11c clone HL3, peridinin chlorophyll protein-Cy5.5-conjugated anti-CD11b clone M1/70, Fluorescein isothiocyanate-conjugated anti-CD86 clone GL1 (all from BD Biosciences), or Alexa Fluor 700-conjugated anti-MHC II clone M5/114.15.2 (eBioscience). Cells were gated on forward scatter (FSC-H/FSC-A) to exclude doublets, on FSC-H and side scatter (SSC-H) to exclude granulocytes, and on Live-Dead Aqua-negative events to exclude dead cells. Percentages of $CD11b^+$ and $CD11c^+$ populations were determined and used to calculate total number of each cell type per organ. For cell proliferation assays, cells were stained with APC-Cy7-conjugated anti-CD4 clone GK1.5 (BD Bioscience), and Live-Dead Aqua. CFSE staining was analyzed on live, $CD4^+$ T cells. All samples were analyzed on a LSR II Optical Bench Flow Cytometer using FACSDiva (BD Biosciences) and Winlist software (Verity Software House).

Proliferation assay

Splenocytes or isolated $CD4^+$ T cells from OT-II/RAG-$1^{-/-}$ mice were stained with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, or CFSE; Invitrogen), according to manufacturer's recommendations using Cell Trace CFSE cell proliferation kit (Invitrogen). CFSE stained cells were co-cultured as described above with either unfractionated splenocytes from RAG-$1^{-/-}$ mice, or with $CD11c^+$ or $CD11b^+$ cells isolated by magnetic cell sorting, as described above. Cells from 72 hour co-cultures were collected and stained with APC-Cy7-conjugated anti-CD4 (BD Bioscience), and Live-Dead Aqua. After gating on live $CD4^+$ T cells, CFSE staining was analyzed by flow cytometry and used to determine the $CD4^+$ T cell proliferation index using Modfit LT Proliferation Wizard (Verity Software House).

Transwell experiment

In the lower chamber of a 24-well Transwell plate (Corning), $CD4^+$ T cells isolated from OT-II RAG-$1^{-/-}$ mice and stained with CFSE were co-cultured with splenocytes from non-infected RAG-$1^{-/-}$ mice, at a ratio of 10:1 in the presence of OVA peptide. Equal numbers of splenocytes from non-infected or infected RAG-$1^{-/-}$ mice were added to the upper chamber. After culture for 72 hours, $CD4^+$ T cell proliferation was analyzed as described above. Controls wells included Transwells with no OVA stimulation and wells with no Transwell.

APC competition assay

$CD4^+$ T cells isolated from OT-II/RAG-$1^{-/-}$ mice were co-cultured with splenic antigen presenting cells from RAG-$1^{-/-}$ mice at a ratio of 1 APC:10 CD4 as described. The APC population consisted of varying proportions of APCs from non-infected mice and infected mice. Supernatants were collected and $CD4^+$ T cell proliferation was analyzed.

Statistical analysis

Due to unequal variances among some of the experimental groups analyzed, non parametric tests were used to test for significant differences between groups. For comparisons between two groups, Mann-Whitney test was used and for comparisons between more than two groups, Kruskal-Wallis test was used followed by Dunn's multiple comparison tests. GraphPad Prism Software Version 5 (GraphPad Software Inc., San Diego, CA) was used to perform all statistical analyses. *P* values of 0.05 or less were considered significant. All experiments were repeated at least twice with 5 animals per group.

Results

Pre-patent schistosome infection impairs T cell production of IFN-γ and proliferation of CD4$^+$ T cells in response to polyclonal stimulation

To determine the effect of pre-patent schistosome infection on the ability of CD4$^+$ T cells to produce cytokines and proliferate during pre-patent infection, we isolated splenic leukocytes from 4 week *S. mansoni* infected wild type mice and assessed their ability to produce IFN-γ in response to antigen-specific and polyclonal stimulation. Cells from infected mice exhibited robust production of IFN-γ in response to soluble worm antigen preparation (SWAP), whereas cells from non-infected mice produced almost no IFN-γ. However, in response to polyclonal T cell stimulation with anti-CD3 antibody, cells from infected mice were significantly impaired in their ability to produce IFN-γ as compared to cells from non-infected mice (Fig. 10A). Furthermore, proliferation of CD4$^+$ T cells from the spleens of infected wild type mice was significantly reduced in response to polyclonal stimulation (Fig. 10B).These data suggest that pre-patent schistosome infection reduces the ability of T cells to produce IFN-γ and specifically impairs CD4$^+$ T cell proliferation in response to polyclonal T cell stimulation.

Pre-patent schistosome infection impairs CD4$^+$ T cell responses to non-schistosome antigens

While pre-patent schistosome infection stimulates the expansion of antigen-specific CD4$^+$ T cells that proliferate and produce cytokines when

subsequently stimulated with schistosome antigens *in vitro* [8], the frequency of schistosome-specific T cells in the T cell population as a whole is suspected to remain low. In contrast, anti-CD3 antibody is an artificial polyclonal stimulus that stimulates all T cells, regardless of specificity. We therefore hypothesized that the reduced responsiveness to anti-CD3 observed in infected mice was due to loss of responsiveness in the T cell population in general, regardless of TCR specificity. To test the effect of infection on T cells with specificity for non-schistosome antigens, we infected TCR-transgenic OT-II/RAG-1$^{-/-}$ mice that only possess OVA-specific CD4^{+} T cells. When splenocytes and hepatic leukocytes were isolated from non-infected OT-II/RAG-1$^{-/-}$ mice, stimulation with OVA peptide induced robust production of IFN-γ and IL-10 (Fig. 11A-D). Stimulation with anti-CD3 antibody also stimulated IFN-γ and IL-10 production by splenocytes (Fig. 11A, 11B), but less robustly than OVA peptide, perhaps indicating that, when all the T cells share the same specificity, cognate stimulation with peptide is a more efficient T cell stimulus than TCR ligation with antibody. In contrast, when splenocytes and hepatic leukocytes isolated from infected OT-II/RAG-1$^{-/-}$ mice were stimulated with OVA peptide, the production of IFN-γ and IL-10 was significantly less when compared to cells from non-infected mice and was comparable to that observed without stimulation (Fig. 11A-D). Likewise, production of IFN-γ and IL-10 in response to anti-CD3 stimulation was also significantly reduced in splenocytes from infected OT-II/RAG-1$^{-/-}$ mice (Fig. 11A, 11B). Additionally, CD4^{+} T cells from the spleens of infected OT-II/RAG-1$^{-/-}$ mice were significantly impaired in their ability to proliferate in response to OVA

peptide or anti-CD3 (Fig. 11E). To test whether schistosome infection directly altered the ability of OT-II T cells to produce cytokines and proliferate, we next isolated splenocytes from infected or non-infected RAG-1 $^{-/-}$ mice and used them as APCs to stimulate naïve CD4^{+} T cells isolated from non-infected OT-II/RAG-1$^{-/-}$ mice. Co-cultures were stimulated with OVA peptide or anti-CD3 and cytokine production and CD4^{+} T cell proliferation was analyzed. When splenocytes from infected mice were used as APCs, production of IFN-γ in response to either OVA peptide or anti-CD3 was significantly reduced compared to co-cultures containing splenocytes from non-infected RAG-1$^{-/-}$ mice (Fig. 11F). Likewise, the proliferation of naïve CD4^{+} T cells in response to both OVA peptide and anti-CD3 stimulation was also significantly impaired in co-cultures with splenocytes from infected mice (Fig. 11G). These data suggest that impairment of CD4^{+} T cell responses by schistosome infection is due to alteration of the ability of accessory cells to stimulate T cells rather than a direct effect on CD4^{+} T cells themselves. These data are consistent with our previous results showing that schistosome infection did not alter the responsiveness of OT-II T cells in OT-II/RAG-1$^{-/-}$mice [49].

Numbers of splenic and hepatic CD11c^{+} and CD11b^{+} cells during pre-patent schistosome infection

To examine whether pre-patent infection induced changes in the number and phenotype of accessory cells that stimulate CD4^{+} T cells, splenic and hepatic

populations of $CD11c^+$ dendritic cells and $CD11b^+$ mononuclear cells in infected and non-infected wild type mice were compared by flow cytometry. There was a significant increase in the total number of dendritic cells in the spleens of infected mice when compared to non-infected controls (Fig. 12A). Furthermore, the total number of dendritic cells expressing MHC II (Fig. 12B) or CD86 (Fig. 12C) in the spleen was also increased by infection, although the former failed to attain statistical significance. For $CD11b^+$ mononuclear cells, the total number of cells in the spleen was significantly increased by infection (Fig. 12D), as was the number of cells expressing MHC II (Fig. 12E) or CD86 (Fig. 12F). Although the total number of $CD11c^+$ dendritic cells and $CD11b^+$ mononuclear cells in the liver was lower than that in the spleen, a similar trend towards higher cell numbers was observed in infected mice (Fig. 12A-F). These data suggest that both $CD11c^+$ and $CD11b^+$ populations of accessory cells respond to schistosome infection and could be implicated in loss of T cell responsiveness in infected mice (Fig. 12B-C).

Pre-patent schistosome infection impairs the ability of $CD11b^+$ cells to stimulate $CD4^+$ cells

To determine whether the loss in T cell stimulatory capacity was associated specifically with either $CD11c^+$ or $CD11b^+$ cells, these populations were isolated from the spleens of infected and non-infected mice and their ability to stimulate OT-II $CD4^+$ T cells *in vitro* was examined. OVA peptide or anti-CD3

stimulation of OT-II T cell/CD11c$^+$ cell co-cultures induced similar levels of IFN-γ production, regardless of whether the CD11c$^+$ cells used as APCs originated from infected or non-infected wild type mice (Fig. 13A). However, when CD11b$^+$ cells isolated from the spleens of infected or non-infected OT-II/RAG-1$^{-/-}$ mice were used as APCs, a significant reduction in the production of IFN-γ in response to anti-CD3 was observed when the CD11b$^+$ cells were isolated from infected mice (Fig. 13B). Likewise, the production of IFN-γ in response to OVA peptide in OT-II T cell/CD11b$^+$ cell co-cultures was diminished when the CD11b$^+$ cells were obtained from infected mice, although this result narrowly avoided attaining statistical significance (P=0.0501; Fig. 13B). Finally, CD11b$^+$ cells from infected mice stimulated significantly less proliferation of OT-II CD4$^+$ T cells than CD11b$^+$ cells from non-infected mice, whether the cells were stimulated with OVA peptide or anti-CD3 stimulation (Fig. 13C). These data suggest that pre-patent schistosome infection impairs the ability of CD11b$^+$ accessory cells to stimulate T cells, but does not alter the T cell stimulatory capacity of CD11c$^+$ dendritic cells.

Impairment of T cell stimulation by pre-patent schistosome infection is independent of arginase, NOS, IL-10, PD-L1 and TGF-β

We hypothesized that the impairment in CD4$^+$ T cell stimulation associated with CD11b$^+$ cells could result from the induction of a suppressor mechanism in CD11b$^+$ cells, as suppressor macrophages that induce T cell anergy have previously been described in schistosome-infected mice [23, 50]. In

these studies, macrophages were found to suppress T cell proliferation through expression of IL-10 or the B7 family member programmed death ligand-1 (PD-L1), also known as B7-H1. Furthermore, other suppressor mechanisms requiring arginase-1 or nitric oxide synthase have been implicated in the suppression of T cell responses by myeloid derived suppressor cells (MDSCs) [51]. To investigate whether these mechanisms mediate T cell suppression induced by schistosome infection, we tested whether inhibitors of these pathways could restore OVA-induced OT-II T cell proliferation in co-cultures where splenocytes from infected RAG-1$^{-/-}$ mice were used as APCs. Nor-NOHA was used to specifically inhibit arginase and L-NMMA was used to inhibit nitric oxide synthase (NOS), both enzymes previously implicated in the inhibition of T cell proliferation by MDSCs [52]. To determine whether the infection-induced impairment in T cell proliferation is mediated by the regulatory cytokine IL-10, IL-10 signaling was inhibited in co-cultures by the addition of anti-IL-10 receptor monoclonal antibody. PD-L1 was inhibited using an anti-PD-L1 monoclonal antibody. None of these treatments restored proliferation to that seen with non-infected APCs. When L-NMMA, anti-IL-10R or anti-PD-L1 were included in co-cultures with splenocytes from infected RAG-1$^{-/-}$ mice, OT-II T cell proliferation was not significantly different from that observed in control cultures without inhibitor, and was still significantly less than the proliferation observed in co-cultures with splenocytes from non-infected mice (Fig. 14A). In cultures containing Nor-NOHA, proliferation of OT-II T cells in response to splenocytes from infected mice was reduced even further when compared to control cultures lacking inhibitor (Fig.

14A). Consistent with these results, no restoration of proliferation was observed when these inhibitors were added to cultures containing un-fractionated splenocytes from infected and non-infected OT-II/RAG-1$^{-/-}$ mice (data not shown). Addition of neutralizing anti-transforming growth factor- β1, β2, β3 (anti-TGF-β1, β2, β3) monoclonal antibody to co-cultures did not restore OT-II T cell proliferation in the presence of splenocytes from infected RAG-1$^{-/-}$ mice (Fig. 14B). These results suggest the impaired stimulation of CD4^{+} T cells is independent of NOS, IL-10, PD-L1 and TGF-β, while arginase activity may support T cell proliferation. Finally, we tested whether addition of exogenous IL-2 could restore OT-II T cell proliferation in the presence of splenocytes from infected RAG-1$^{-/-}$ mice, as exogenous IL-2 has previously been shown to restore proliferation in anergic T cells [14, 53, 54]. However, the addition of IL-2 to co-cultures did not lead to restoration of T cell proliferation in the presence of infected RAG-1$^{-/-}$ splenocytes (Fig. 14C). Indeed, the addition of exogenous IL-2 to co-cultures with non-infected RAG-1$^{-/-}$ splenocytes inhibited proliferation, such that the level of proliferation was no longer significantly different than in co-cultures containing infected RAG-1$^{-/-}$ splenocytes (Fig. 14C). Taken together, these data suggest that the loss of T cell proliferation in the presence of splenocytes from infected RAG-1$^{-/-}$ mice is mediated by a mechanism that is distinct from mechanisms of T cell suppression previously identified in schistosome-infected mice, including anergy induction.

T cell suppression induced by pre-patent schistosome infection is cell contact-dependent

As our data failed to support a role for several established soluble mediators in limiting T cell responsiveness, we next tested whether the mechanism of T cell suppression was mediated by a soluble, diffusible mediator or required cell contact, using cell-impermeable Transwell inserts. The lower chamber contained splenocytes from non-infected RAG-1$^{-/-}$ mice plus OVA peptide and CFSE-labeled OT-II T cells. Splenocytes from either infected or non-infected RAG-1 $^{-/-}$ mice were then added to the upper chamber and the proliferation of the T cells in the lower chamber was assessed following three days of culture. Regardless of whether the upper chamber contained splenocytes from infected or non-infected RAG-1$^{-/-}$ mice, there was no difference in the proliferation of the OT-II T cells in the lower chamber (Fig. 15A), suggesting that the suppression of T cell proliferation by splenocytes from infected mice is not caused by the production of a diffusible inhibitor.

To test whether splenocytes from infected RAG-1$^{-/-}$ mice could inhibit the stimulatory capacity of splenocytes from non-infected RAG-1$^{-/-}$ mice, we performed competition assays, where splenocytes from infected and non-infected RAG-1$^{-/-}$ mice were mixed in varying proportions and used as APCs to stimulate OT-II T cells. When splenocytes from infected mice comprised 10-75 % of the APCs, there was no significant difference in proliferation of the T cells when compared to T cells cultured in the presence of non-infected RAG-1$^{-/-}$ splenocytes alone (Fig. 15B). Indeed, no significant impairment in T cell

proliferation was detected until the infected RAG-1$^{-/-}$ splenocytes outnumbered those from non-infected mice by a ratio of 3:1, when proliferation levels were similar to those obtained with infected RAG-1$^{-/-}$ splenocytes alone (Fig. 15B). Together, these data suggest that T cell suppression by splenocytes from infected mice is not mediated by a soluble factor, but instead requires cell-to-cell contact. Furthermore, these data suggest that, when compared cell-for-cell, splenocytes from infected mice cannot suppress the T cell proliferation stimulated by splenocytes from non-infected mice until they outnumber cells from non-infected mice by a ratio of more than 3:1.

Suppression of IL-12 expression may contribute to impairment of T cell responses during pre-patent schistosome infection

As T cell suppression by splenocytes from infected RAG-1$^{-/-}$ mice required cell contact and did not occur unless splenocytes from infected mice outnumbered those from non-infected mice, we hypothesized that the T cell suppression resulted from a loss in T cell stimulatory capacity rather than production of a suppressive mediator. This hypothesis is also consistent with our finding that splenocytes from infected mice inhibited T cell proliferation only when they outnumbered splenocytes from non-infected mice, because it is presumably only when they out-compete competent APCs for interactions with T cells that the inhibition would be detectable. To test this hypothesis, we examined infected mice for evidence of impaired T cell stimulatory capacity. Proliferation of T cells

and their production of IFN-γ is potently stimulated by IL-12, a T cell stimulatory factor produced by $CD11b^+$ and $CD11c^+$ APCs. We therefore hypothesized that defective T cell stimulation by APCs from infected mice may be due to a failure to produce adequate IL-12. Consistent with this hypothesis, the expression of IL-12p35 was significantly down-regulated in the spleen during pre-patent infection (Fig. 16A). A similar trend was observed in liver tissue, although the difference was not statistically significant. As exogenous IL-12 has been shown to reverse anergy and restore T cell production of IFN-γ [55, 56], we tested whether addition of IL-12 to co-cultures containing infected APCs could restore cytokine production and T cell proliferation. Adding exogenous IL-12 did not restore proliferation and in fact appeared to further impair proliferation of OT-II T cells regardless of whether APCs from infected or non-infected mice were used (Fig. 16B). However, addition of IL-12 significantly increased IFN-γ production by OVA peptide-stimulated OT-II cells co-cultured with splenocytes from either infected or non-infect mice (Fig. 16C), to the extent that there was no longer any difference when APCs from infected and non-infected mice were compared. These data suggest that IL-12 deficiency in innate APCs during pre-patent infection contributes to impaired T cell cytokine production. However, there are likely other factors that contribute to the mechanism of T cell suppression, as IL-12 did not restore proliferation.

Discussion

Previous studies have shown that during primary schistosome infection, migration of juvenile worms from the skin to the portal vasculature is accompanied by systemic induction of antigen-specific $CD4^+$ T cells that produce IFN-γ, IL-4 and IL-10 in response to schistosome worm antigens. Furthermore, our data show that $CD4^+$ T cells are the predominant source of these cytokines prior to the onset of egg-laying ([8] and Fig. 6). Interestingly, however, we show here for the first time that induction of schistosome-specific $CD4^+$ T cell responses during pre-patent infection is accompanied by loss of $CD4^+$ T cell responsiveness to polyclonal stimulation by anti-CD3 antibody. In non-infected mice, stimulation of splenocytes with anti-CD3 antibody produces robust IFN-γ production and $CD4^+$ T cell proliferation (Fig. 10), while stimulating little IL-4 or IL-10 production (Fig. 17). In contrast, both IFN-γ production and $CD4^+$ T cell proliferation in response to anti-CD3 stimulation were impaired in cells from mice with pre-patent schistosome infection (Fig. 10), with no detectable increase in production of IL-4 or IL-10 (Fig. 17). Thus, induction of $CD4^+$ T cell responses to schistosome antigens was accompanied by a decrease in $CD4^+$ T cell responsiveness to polyclonal stimulation. These results are in agreement with previously published findings, which showed a reduction in IFN-γ production by T cells in response to polyclonal stimulation in wild type mice with a patent schistosome infection [31]. However, to our knowledge, our data are the first to suggest a loss of T cell responsiveness is associated with pre-patent infection.

Our data suggest that, during pre-patent infection, induction of $CD4^+$ T cell responses to schistosome antigens is accompanied by a loss of $CD4^+$ T cell responsiveness to other non-schistosome antigens. Furthermore, our findings raise the possibility that schistosome-specific $CD4^+$ T cell responses may be implicated in the regulation of responses to other non-schistosome antigens. To address both of these possibilities, we examined T cell responsiveness in OT-II/RAG-$1^{-/-}$ that are devoid of all T cells, except for a monospecific population of $CD4^+$ T cells that recognize the same $OVA_{323-339}$ peptide in the context of the mouse I-A^b MHC class II molecule. Thus, these animals allowed us to examine $CD4^+$ T cell responses to an irrelevant bystander antigen (OVA) in a context where $CD4^+$ T cell responses to the parasite are absent. When splenocytes from infected OT-II/RAG-$1^{-/-}$ were stimulated with either OVA peptide or anti-CD3, we again saw a significant defect in cytokine production and $CD4^+$ T cell proliferation, suggesting that (i) pre-patent schistosome infection does indeed result in loss of $CD4^+$ T cell responsiveness to non-parasite antigens, and (ii) that this loss of responsiveness is independent of adaptive responses to schistosomes. Our findings suggest that, during pre-patent infection there is impairment of $CD4^+$ T cell responsiveness, but that this loss of responsiveness occurs as a result of alterations in innate immune function rather than a direct effect on T cells. Indeed, we have previously shown that isolated OT-II $CD4^+$ T cells respond equally well to OVA or anti-CD3 stimulation, regardless of whether they are isolated from infected or non-infected mice [57]. Confirming the role of innate cells in modulating T cell responsiveness, the loss of cytokine production

and proliferation was recapitulated when OT-II T cells from non-infected mice were stimulated with splenocytes from infected RAG-1$^{-/-}$ mice. Therefore our data suggest that pre-patent schistosome infection induces changes in innate cells that impair their ability to stimulate CD4^{+} T cell responses.

Professional APCs of the innate immune system consist of two main populations belonging to the mononuclear phagocyte system, namely dendritic cells and monocytes/macrophages. In particular, dendritic cells are known to have a vital role in the immune response to schistosomes and several reports highlight the importance of CD11c^{+} dendritic cells in priming Th2 cell responses during schistosome infection [58-61]. Liver macrophages have also been implicated in inducing the activation and type 2 differentiation of CD4^{+} T cells during schistosome infection [62]. CD4^{+} T cell stimulation by APCs requires expression of MHC class II-peptide complexes and co-stimulatory ligands [63-65] and reduced expression of these molecules by APCs during T cell priming can cause T cells to become unresponsive [54]. Our data did not provide any evidence that pre-patent infection impaired the expression of MHC class II or CD86 by either APC population. In contrast, when CD11c^{+} and CD11b^{+} cells were isolated from infected mice and their ability to stimulate CD4^{+} T cells in vitro was compared, we found that a significant defect in T cell stimulation was associated with the CD11b^{+} cells and not the CD11c^{+} cells. These observations suggest that CD11c^{+} dendritic cells are functionally intact and that impairment of DC function is not the cause of reduced T cell responsiveness during pre-patent infection. Rather, our findings suggest that impairment of CD11b^{+} APC function

is responsible, at least in part, for the loss of T cell responsiveness seen during pre-patent infection. These findings are consistent with previous evidence supporting a regulatory role for macrophages during schistosome infection. For example, a schistosome polysaccharide with immunomodulatory properties was found to mediate its effects by eliciting a population of suppressor macrophages that suppressed $CD4^+$ T cell proliferation [23]. In another study, macrophages from infected mice were shown to be responsible for the induction of T cell anergy during schistosome infection [50].

In these previous studies, the mechanisms implicated in the suppression of T cell proliferation by macrophages were the production of NO [23] and the expression of PD-L1 (B7-H1) [50]. In contrast, our data suggest that neither of these mechanisms accounts for the suppression of T cell responses in our system. Minor differences in the methods employed may account for some of the discrepancies between our results and those previously reported, including our use of schistosome infection rather than glycoconjugate exposure [23], and our use of naïve monospecific T cells and peptide antigen (OVA) rather than anti-CD3 stimulation of polyclonal T cell populations with presumably mixed naïve, activated and memory phenotypes [50]. However, there are other mechanisms by which $CD11b^+$ cells may suppress T cell proliferation. For example arginase, which together with nitric oxide synthase participates in arginine metabolism, has been implicated in the suppression of T cell proliferation by myeloid-derived suppressor cells [51]. Furthermore, the regulatory cytokine IL-10 is produced by both T cell and non-T cell sources during schistosome infection [34], and has

been shown to inhibit the priming of Th1 responses by dendritic cells [66]. Finally, TGF-β, a regulatory cytokine produced by macrophages and other cells, was shown to suppress T cell responses in schistosome infection [67]. However, interference with each of these other regulatory pathways also failed to restore T cell proliferation, suggesting that the mechanism responsible for T cell suppression we observed is distinct from mechanisms of macrophage-mediated T cell suppression and anergy induction previously identified in schistosome infection. Further underlying this distinction, we found that provision of exogenous IL-2, which has been shown to reverse clonal anergy in $CD4^+$ T cells [53], also did not restore T cell proliferation in the presence of splenocytes from infected mice, suggesting the T cells are not subject to anergy induction.

As we could find no evidence that any of these candidate mechanisms were significantly influencing APC function, we questioned whether the observed impairment was cell contact-dependent or -independent and whether infection causes innate APCs to produce a diffusible suppressive or inhibitory molecule. Consistent with data suggesting that the T cell suppression we observed was not mediated by the diffusible factors IL-10 and TGF-β, we found evidence that the T cell suppression was also contact-dependent, as no impairment of T cell proliferation was observed unless the T cells were mixed directly with splenocytes from infected mice in the same well. Furthermore, T cell proliferation was not significantly impaired until splenocytes from infected mice outnumbered those from non-infected mice by a ratio of 3:1. These findings suggest the “suppressor” cells induced by infection are not potent suppressors of T cells.

Rather, these cells may simply be impaired in their capacity to stimulate T cells, such that T cell suppression only occurs when they are present in sufficient numbers to out-compete competent APCs. In support of a model whereby APC stimulatory capacity is lost rather than an active suppressor mechanism induced, we found that pre-patent schistosome infection was associated with a significant decrease in the baseline expression of IL-12, a critical stimulator of T cell proliferation and IFN-γ production. Indeed, addition of exogenous IL-12 to T cell-splenocyte co-cultures stimulated robust IFN-γ production, even when splenocytes from infected mice were used as APCs. Consistent with a potential role for IL-12 downregulation in T cell suppression, previous studies showed that schistosome egg antigens suppressed IL-12 production by LPS-stimulated dendritic cells [68]. Furthermore, a recent study demonstrated that IL-12 production was suppressed in the intestines of mice harboring patent schistosome infections, by a mechanism that requires arginase I expression [69]. However, schistosome-induced impairment of IL-12 expression is likely not the only factor that contributes to loss of T cell responsiveness during pre-patent infection, as exogenous IL-12 did not restore T cell proliferation in T cell-splenocyte co-cultures. Thus, we propose a model where pre-patent schistosome infection leads to a loss of T cell stimulatory capacity amongst innate accessory cells, and therefore to T cell suppression, by a mechanism that involves, but is not limited to, inhibition of IL-12 expression.

In summary, we have shown that $CD11b^+$ cells exposed to pre-patent schistosome infection are impaired in their ability to stimulate $CD4^+$ T cells and

that this may contribute to an overall reduction of T cell responsiveness during pre-patent infection. Furthermore, we have provided evidence to suggest that infection-induced impairment of IL-12 production contributes to this loss of T cell stimulatory capacity. Future studies will seek to identify other aspects of T cell stimulation that are suppressed by pre-patent schistosome infection and will examine the mechanisms by which juvenile schistosomes induce these suppressive effects. An understanding of the host-parasite interactions that lead to immune hyporesponsiveness may identify opportunities to selectively stimulate immunity against helminths in infected and susceptible hosts.

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Figure 10. Pre-patent schistosome infection impairs T cell production of IFN-γ and proliferation of $CD4^+$ T cells in response to polyclonal stimulation

A, Splenocytes isolated from 4 week *S. mansoni*-infected or non-infected wild type mice were cultured *in vitro* for 72 hours without antigen, with SWAP or with anti-CD3 antibody. The concentration of IFN-γ was measured from cell culture supernatant by ELISA. Data shown are mean +/- SEM. B, Splenocytes isolated from 4 week-infected or non-infected wild type mice were stained with CFSE and cultured for 72 hrs without antigen or with αCD3 antibody. Cells were recovered from culture, stained for surface markers, then CFSE dilution due to cell division was analyzed by flow cytometry and proliferation index calculated. Horizontal bars represent mean value of 5 independent mice. Data are representative of 3 independent experiments. No Ag, no antigen; SWAP, soluble worm antigen preparation; αCD3, anti-CD3 antibody.

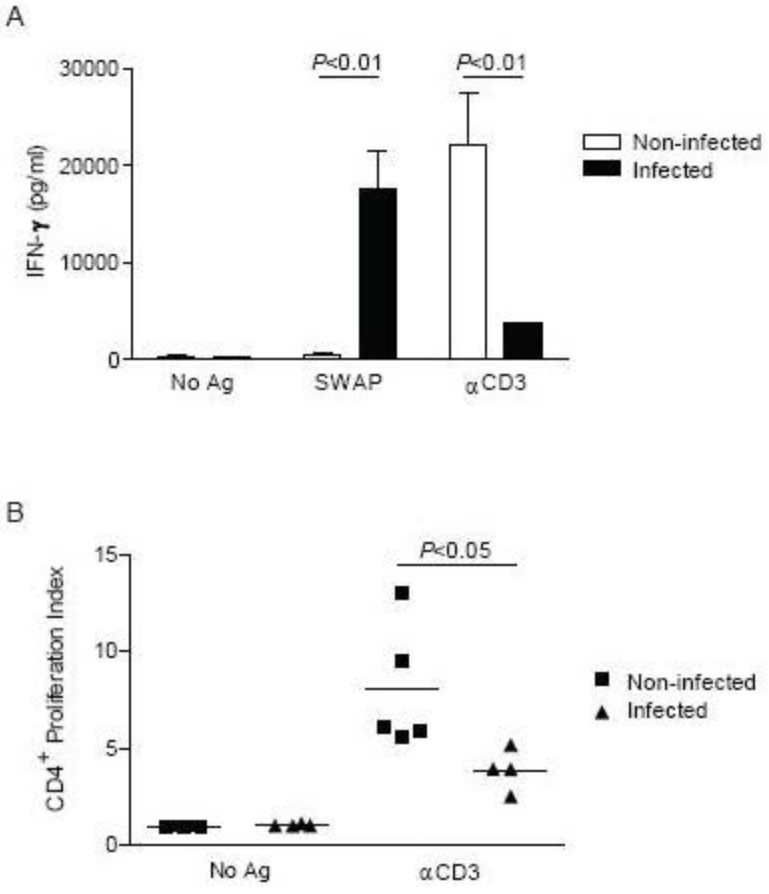

A
P<0.01
P<0.01
30000
20000
10000
0
IFN-γ (pg/ml)
Non-infected
Infected
No Ag
SWAP
αCD3
B
P<0.05
15
10
5
0
CD4+ Proliferation Index
Non-infected
Infected
No Ag
αCD3

Figure 11. Pre-patent schistosome infection impairs $CD4^+$ T cell responses to non-schistosome antigens

A, Splenocytes (A-B) or hepatic leukocytes (C-D) isolated from 4 week *S. mansoni*-infected or non-infected OT-II/RAG-$1^{-/-}$ mice were cultured *in vitro* for 72 hours without antigen, or in the presence of OVA peptide or anti-CD3 antibody. The concentration of IFN-γ (A,C) or IL-10 (B,D) in the cell culture supernatant was measured by ELISA. Data shown are mean +/- SEM. E, Splenocytes were isolated from 4 week-infected or non-infected OT-II/RAG-$1^{-/-}$ mice, stained with CFSE, and cultured for 72 hrs with OVA peptide or anti-CD3 stimulation. Cells were recovered from culture, stained for surface markers, then CFSE dilution due to cell division was analyzed by flow cytometry and proliferation index calculated. Horizontal bars represent mean value of 5 independent mice. F, Splenocytes were isolated from 4 week *S. mansoni*-infected or non-infected RAG-$1^{-/-}$ mice; $CD4^+$ T cells were isolated from non-infected OT-II/RAG-$1^{-/-}$ mice and stained with CFSE. Cells were co-cultured for 72 hours without antigen or in the presence of OVA peptide or anti-CD3 antibody. The concentration of IFN-γ in cell culture supernatants was measured by ELISA (F) and $CD4^+$ T cell proliferation was analyzed by flow cytometry (E). Data are representative of at least 3 independent experiments. No Ag, no antigen; OVA, OVA peptide; αCD3, anti-CD3.

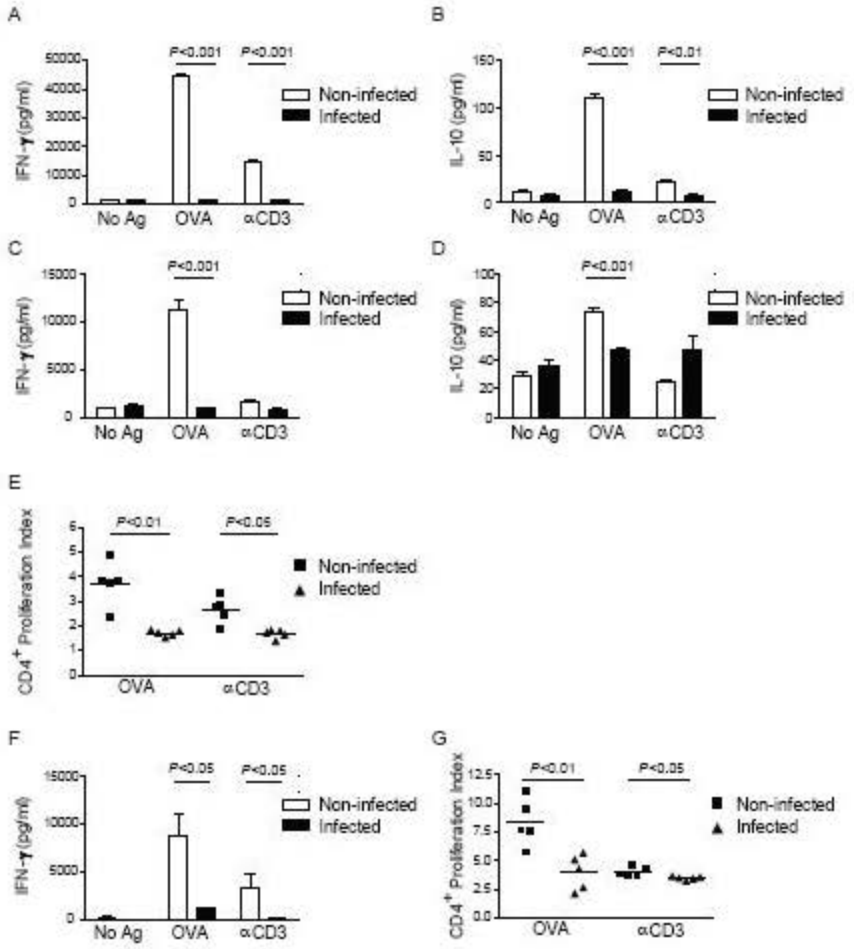

A
B
C
D
E
F
G
IFN-γ (pg/ml)
IL-10 (pg/ml)
CD4+ Proliferation Index
P<0.001
P<0.01
P<0.05
Non-infected
Infected
No Ag
OVA
αCD3

Figure 12. Numbers of splenic and hepatic $CD11c^+$ and $CD11b^+$ cells during pre-patent schistosome infection

A, Flow cytometric analysis of cells isolated from the spleen or liver of 4 week *S. mansoni* infected or non-infected mice. Cells were gated and the absolute number of cells of each phenotype was calculated as described in Methods. Total numbers of $CD11c^+$ (A), $CD11c^+$ MHC class II^+ (B) or $CD11c^+$ CD86 (C) cells per organ. Total numbers of $CD11b^+$ (D), $CD11b^+$ MHC class II^+ (E) or $CD11c^+$ $CD86^+$ (F) cells per organ. Horizontal bars represent the mean of 5 independent mice.

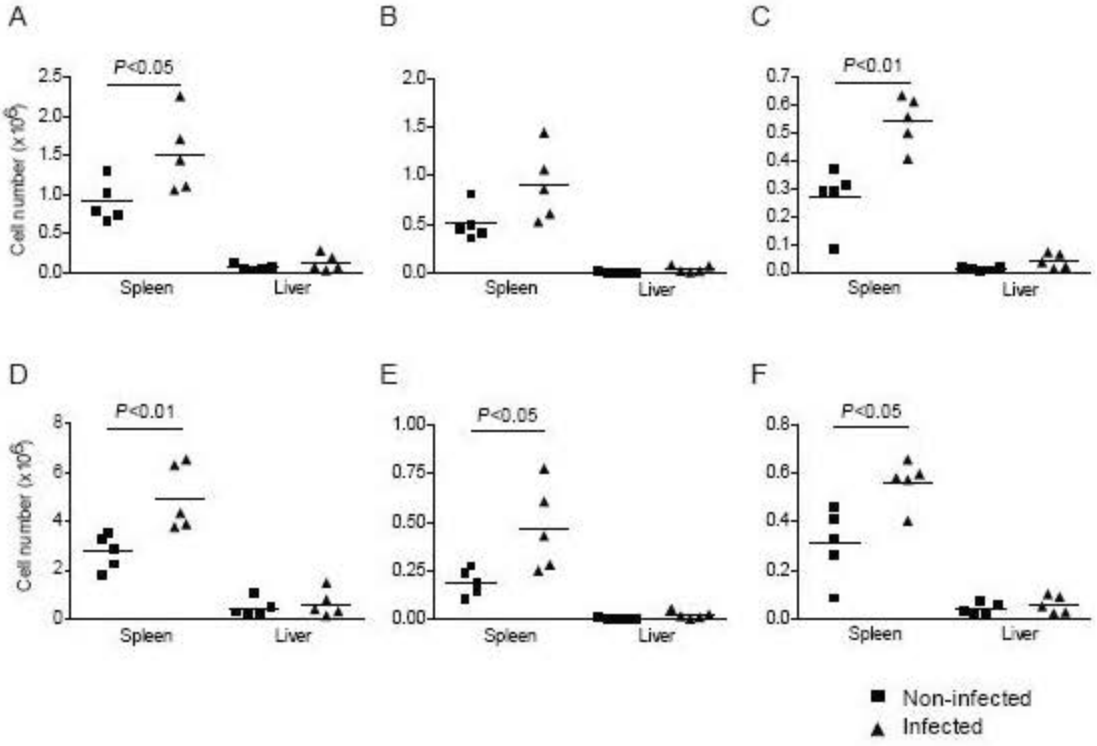

A
B
C
D
E
F
P<0.05
P<0.01
P<0.05
P<0.01
P<0.05
Cell number (x10^6)
Spleen
Liver
Non-infected
Infected

Figure 13. Pre-patent schistosome infection impairs the ability of $CD11b^+$ cells to stimulate $CD4^+$ cells

A, $CD11c^+$ dendritic cells isolated from the spleen of 4 week *S. mansoni*-infected or non-infected wild type mice and $CD4^+$ T cells isolated from non-infected OT-II/RAG-$1^{-/-}$ mice were co-cultured for 72 hours without antigen, with OVA peptide or with anti-CD3 antibody. The concentration of IFN-γ in the cell culture supernatants was measured by ELISA. B, $CD11b^+$ cells were isolated from the spleens of 4 week *S. mansoni*-infected or non-infected OT-II/RAG-$1^{-/-}$ mice; $CD4^+$ T cells were isolated from non-infected OT-II/RAG-$1^{-/-}$ mice, and stained with CFSE. Cells were then co-cultured for 72 hours without antigen, with OVA peptide or with anti-CD3 antibody. The concentration of IFN-γ in the cell culture supernatants was measured by ELISA. Data shown are mean +/- SEM. C, $CD4^+$ T cell proliferation from co-cultures described in (B) was analyzed by flow cytometry and proliferation index calculated. Horizontal bars represent mean of 5 independent mice. No Ag, no antigen; OVA, OVA peptide; αCD3, anti-CD3.

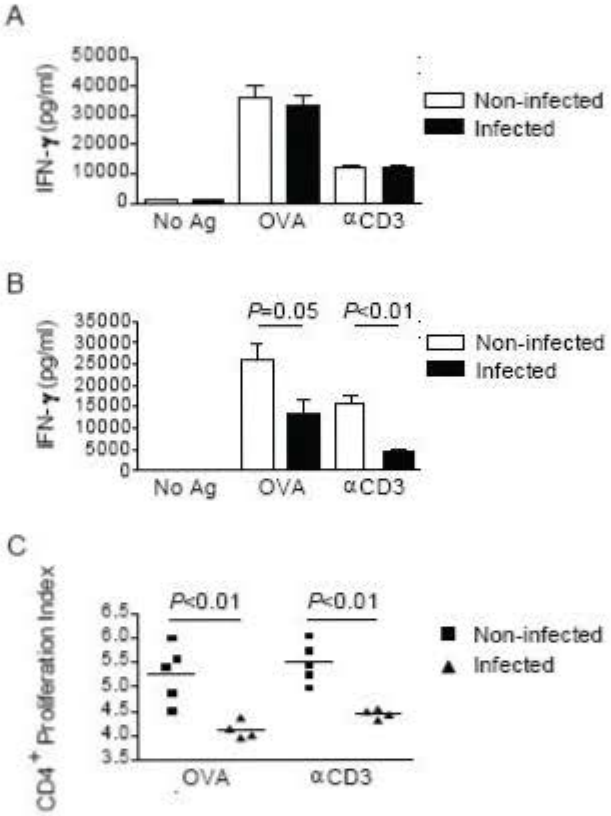

A
IFN-γ (pg/ml)
50000
40000
30000
20000
10000
0
No Ag
OVA
αCD3
Non-infected
Infected
B
P=0.05
P<0.01
IFN-γ (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
No Ag
OVA
αCD3
Non-infected
Infected
C
CD4+ Proliferation Index
P<0.01
P<0.01
6.5
6.0
5.5
5.0
4.5
4.0
3.5
OVA
αCD3
Non-infected
Infected

Figure 14. Impairment of T cell stimulation by pre-patent schistosome infection is independent of arginase, NOS, IL-10, PD-L1 and TGF-β

A, Splenocytes isolated from 4 week *S. mansoni*-infected or non-infected RAG-1$^{-/-}$ mice co-cultured with CFSE-stained CD4^{+} T cells isolated from non-infected OT-II/RAG-1$^{-/-}$ mice for 72 hrs with OVA peptide stimulation in the presence of Nor-NOHA, L-NMMA, anti-IL-10R or anti-PD-L1. Cells recovered from culture were stained for surface markers then analyzed for CD4^{+} T cell proliferation by flow cytometry. Proliferation Index was calculated. Horizontal bars represent mean of 5 independent mice. B, In a replicate, independent experiment, cells were isolated as in (A) and cultured in the presence of OVA peptide stimulation with anti-TGF-β1, β2, β3 antibody (B) or recombinant IL-2 (C) and. CD4^{+} T cell proliferation was analyzed by flow cytometry. Horizontal bars represent mean of 5 independent mice. Data are representative of at least 3 independent experiments. Nor-NOHA, N^{ω}-hydroxy-nor-Arginine; L-NMMA, L-N^{G}-monomethyl Arginine citrate; αIL-10R, anti-IL-10 receptor monoclonal antibody; αPD-L1, anti-PD-L1 monoclonal antibody; αTGF-β, anti-TGF-β1, β2, β3 monoclonal antibody.

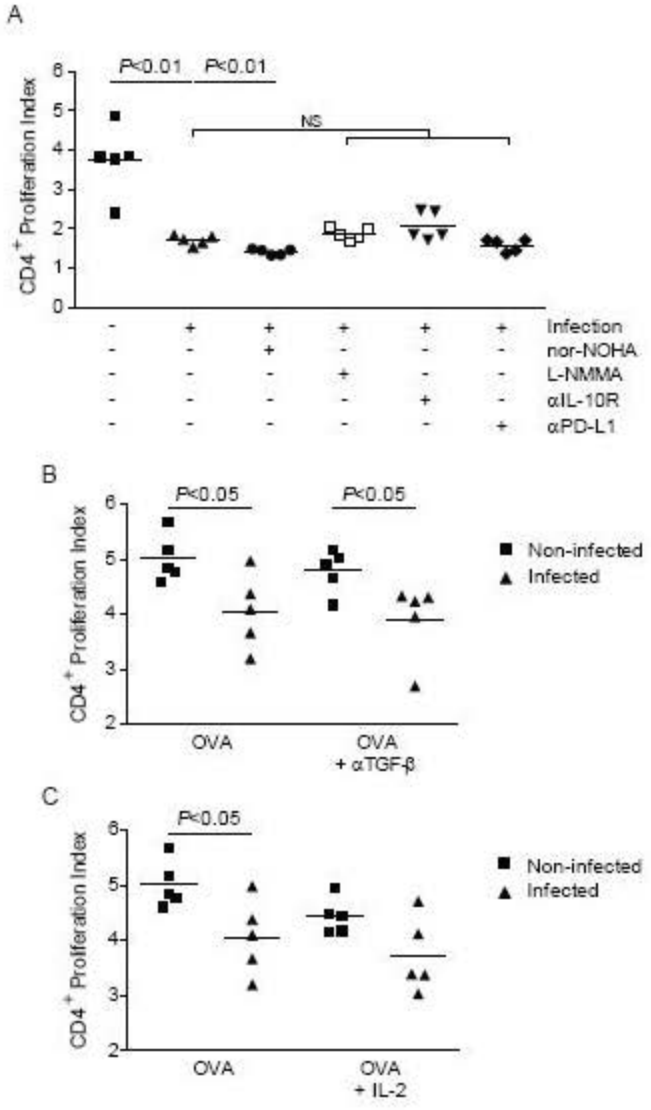

A
P<0.01
P<0.01
NS
CD4+ Proliferation Index
Infection
nor-NOHA
L-NMMA
αIL-10R
αPD-L1
B
P<0.05
P<0.05
Non-infected
Infected
OVA
OVA + αTGF-β
C
P<0.05
Non-infected
Infected
OVA
OVA + IL-2

Figure 15. T cell suppression induced by pre-patent schistosome infection is cell contact-dependent

A, Transwell experiment was performed where the lower chamber contained splenocytes isolated from non-infected RAG-$1^{-/-}$ mice co-cultured with CFSE-stained $CD4^+$ T cells isolated from non-infected OT-II/RAG-$1^{-/-}$ mice. Upper chamber contained splenocytes isolated from 4 week *S. mansoni*-infected or non-infected RAG-$1^{-/-}$ mice. Cells were co-cultured for 72 hours in the presence of OVA peptide and $CD4^+$ T cell proliferation was analyzed by flow cytometry. Horizontal bars represent mean of 5 independent mice. B, Competition assay was performed where varying proportions of splenocytes isolated from 4 week *S. mansoni*-infected or non-infected RAG-$1^{-/-}$ mice were co-cultured with CFSE stained $CD4^+$ T cells isolated from non-infected OT-II/RAG-$1^{-/-}$ mice. $CD4^+$ T cell proliferation was analyzed by flow cytometry. Horizontal bars represent mean of 5 independent mice. APCs, antigen presenting cell.

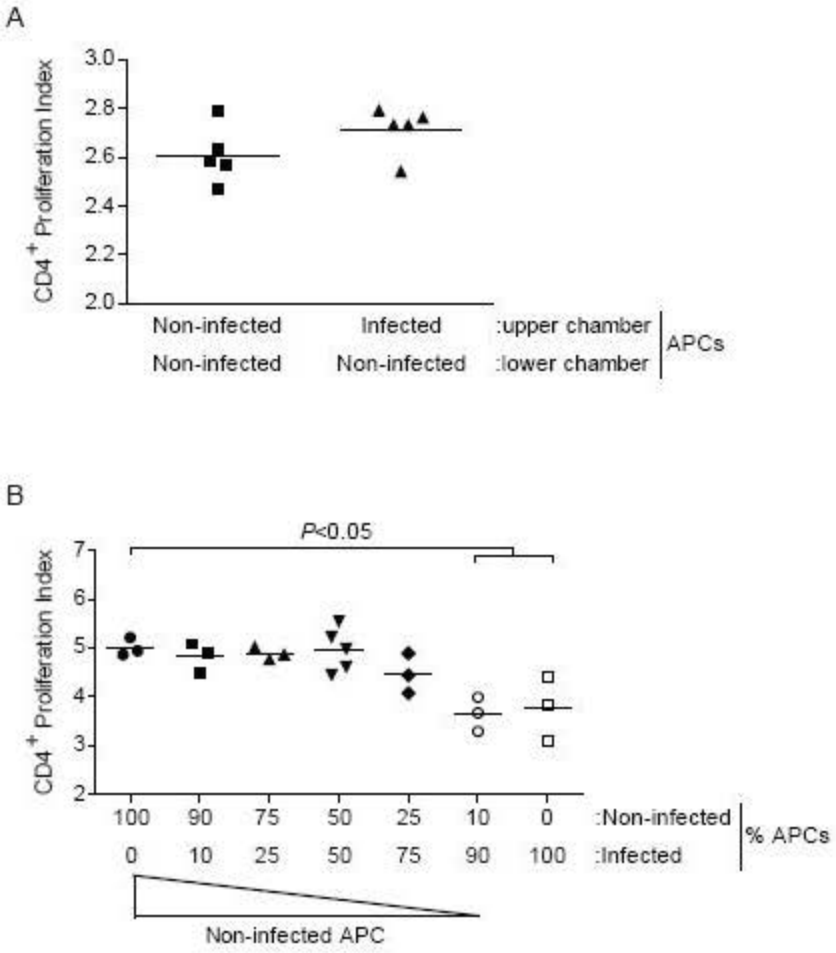

A
CD4+ Proliferation Index
3.0
2.8
2.6
2.4
2.2
2.0
Non-infected
Infected
:upper chamber
Non-infected
Non-infected
:lower chamber
APCs
B
P<0.05
CD4+ Proliferation Index
7
6
5
4
3
2
100 90 75 50 25 10 0 :Non-infected
0 10 25 50 75 90 100 :Infected
% APCs
Non-infected APC

Figure 16. Suppression of IL-12 expression may contribute to impairment of T cell responses during pre-patent schistosome infection

A, Taqman real-time PCR gene expression assay for IL-12p35 was performed on RNA isolated from tissue of the spleen or liver of 4 week *S. mansoni*-infected or non-infected mice wild type mice. Fold change was calculated using the comparative C_T method and GAPDH as the endogenous control. B and C, Splenocytes isolated from 4 week *S. mansoni* infected or non-infected RAG-$1^{-/-}$ mice or and CFSE-stained $CD4^+$ T cells isolated from non-infected OT-II/RAG-$1^{-/-}$ mice were co-cultured for 72 hours without antigen, with indicated combinations of OVA peptide and IL-12, or with anti-CD3 antibody. $CD4^+$ T cell proliferation was analyzed by flow cytometry (B) and the concentration of IFN-γ in the cell culture supernatants was measured by ELISA (C). Horizontal bars represent mean of 3 independent mice (A, B). Data shown are mean +/- SEM (C). No Ag, no antigen; OVA, OVA peptide; αCD3, anti-CD3 antibody.

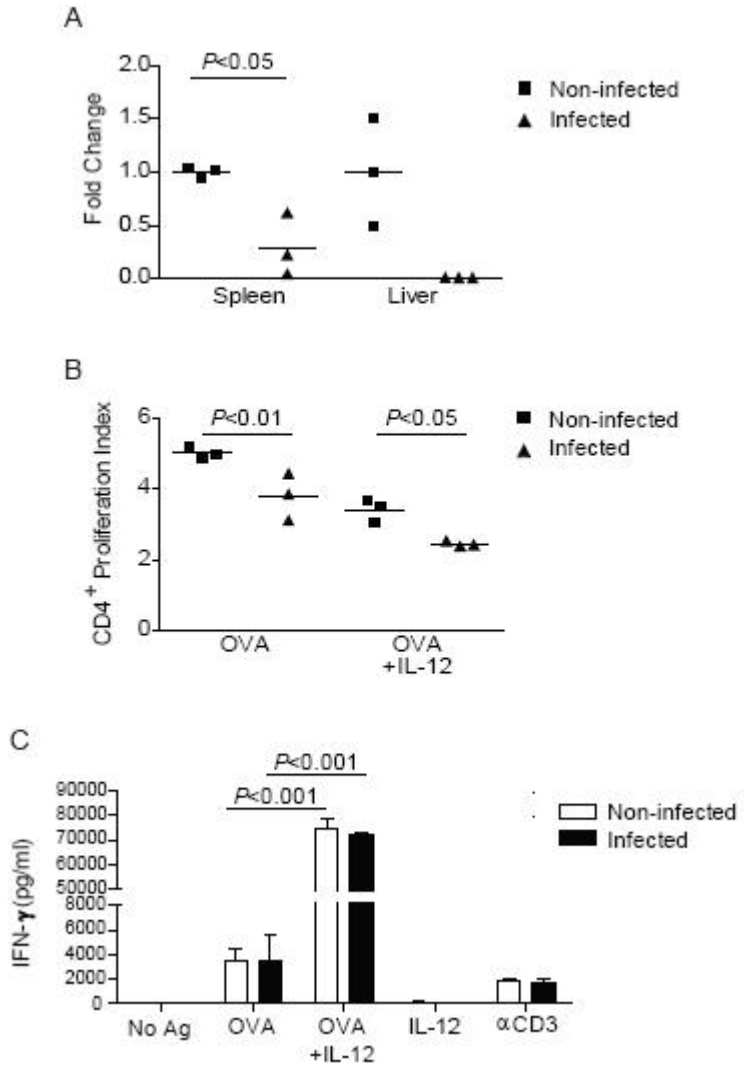
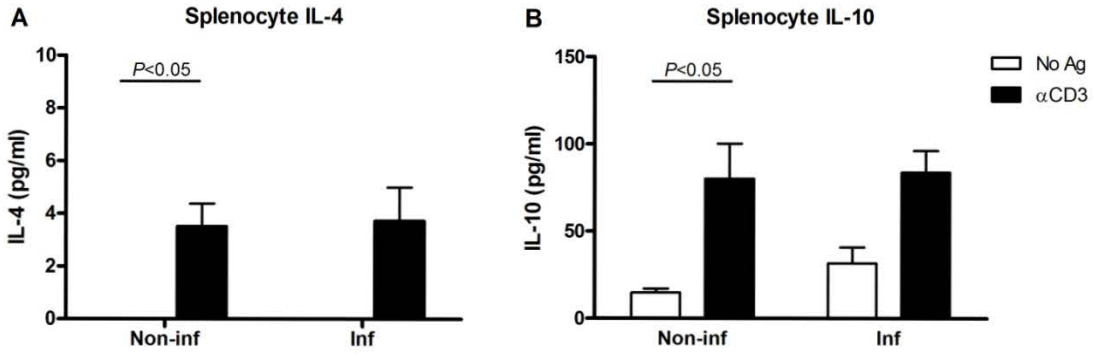

A
P<0.05
Fold Change
2.0
1.5
1.0
0.5
0.0
Spleen
Liver
Non-infected
Infected
B
P<0.01
P<0.05
CD4+ Proliferation Index
6
4
2
0
OVA
OVA +IL-12
Non-infected
Infected
C
P<0.001
P<0.001
IFN-γ (pg/ml)
90000
80000
70000
60000
50000
8000
6000
4000
2000
0
No Ag
OVA
OVA +IL-12
IL-12
αCD3
Non-infected
Infected

Figure 17. Pre-patent schistosome infection does not induce the production of IL-4 or IL-10 in response to polyclonal stimulation

A, Splenocytes isolated from 4 week *S. mansoni*-infected or non-infected wild type mice were cultured *in vitro* for 72 hours without antigen or with anti-CD3 antibody. The concentration of IL-4 (A) or IL-10 (B) was measured from cell culture supernatant by ELISA. Data shown are mean +/- SEM. αCD3, anti-CD3 antibody.


A
Splenocyte IL-4
P<0.05
IL-4 (pg/ml)
10
8
6
4
2
0
Non-inf
Inf
B
Splenocyte IL-10
P<0.05
IL-10 (pg/ml)
150
100
50
0
Non-inf
Inf
No Ag
αCD3

Chapter 4

Additional Experiments and Discussion

Relevance of Our Work

The burden of disease associated with schistosomiasis is quite significant, with over 200 million people affected throughout the tropics and subtropics [1]. The burden to the individual host is considerable as female schistosome worms can produce hundreds to thousands of eggs per day and adult worms can survive within the host for prolonged periods of time [2]. In fact, it has been suggested that the disease burden for schistosomiasis has been underestimated and that the real burden is much higher than previous estimates [3]. Not only is there a physical burden to the host, but there is also an economic burden associated with disease in endemic communities. Illness caused by schistosomiasis leads to a reduction in the workforce number and productivity, and a loss of income for the individual. These long term disabilities also increase the difficulty of combating rural poverty, impacting the economy at large. School age children are especially affected by schistosomiasis. This age group has the highest prevalence of human schistosomiasis and also the highest morbidity and mortality associated with disease [4]. Children have been shown to suffer from growth retardation [5] and developmental delays [6] due to the disease, with the possibility that these difficulties will be a lifelong struggle for these children. This astonishing burden connected with schistosomiasis, alone, provides rationale for research of the disease.

There are additional reasons to study schistosomiasis, including the need for new drugs and the requirement for an effective vaccine strategy. The mainstay of treatment for schistosomiasis is praziquantel, which is safe and

effective, but the absence of alternative drugs highlights the risk for development of resistance to praziquantel. The danger of resistance is even more pertinent in endemic areas where re-infection is almost impossible to prevent because people living and working in endemic areas are constantly exposed to infection through their daily activities and occupation [7]. Vaccine development is just as much of a quagmire. Recent attempts to formulate vaccines for schistosomiasis have failed to attain protective efficacy [8] that is improved over the attenuated cercarial vaccine [9], one of the first vaccine strategies developed. What makes drug and vaccine development for schistosomiasis even more difficult is that natural infection does not lead to the development of sterile or acquired immunity. In some cases, people in endemic areas can develop resistance to re-infection after several rounds of drug treatment, however this does not prevent infection but merely causes an increase in the length of time from drug cure until the individual becomes egg positive again [10]. Additionally, the development of this form of resistance to re-infection was found to be dependent on the immune status of the individual [10].

Co-infection and Relevance of Regulatory Response

The immune status of people living in areas endemic for schistosomiasis is complicated by the presence of co-infecting pathogens. In reality, people living in endemic areas are more likely to be co-infected by several pathogens than to have only one infection [11]. This state of polyparasitism is associated with an

exacerbated disease burden and with increased morbidity, including iron deficiency anemia, growth retardation and impaired cognition [12]. Some infections that are commonly found co-endemic with schistosomiasis include malaria caused by *Plasmodium falciparum [13]*, hookworm infection [14] and salmonellosis caused by *Salmonella* species [15, 16]. A compounding factor for morbidity due to co-infection with schistosomiasis and malaria, or salmonellosis, is that these pathogens can cause disease in the same host organ, primarily the liver. Schistosomiasis has also been shown to be associated with HIV co-infection [17] and monkeys with pre-existing schistosome infection have been found to be more susceptible to infection with simian HIV [18]. As mentioned previously, children are especially susceptible to the higher morbidity associated with co-infections. Children co-infected with schistosomiasis and malaria exhibit exacerbated splenomegaly [19] and co-infection in children is also known to mediate immunological alterations in certain T lymphocyte subsets that may be associated with immuno-regulation [20].

Co-infection with *Salmonella typhimurium*

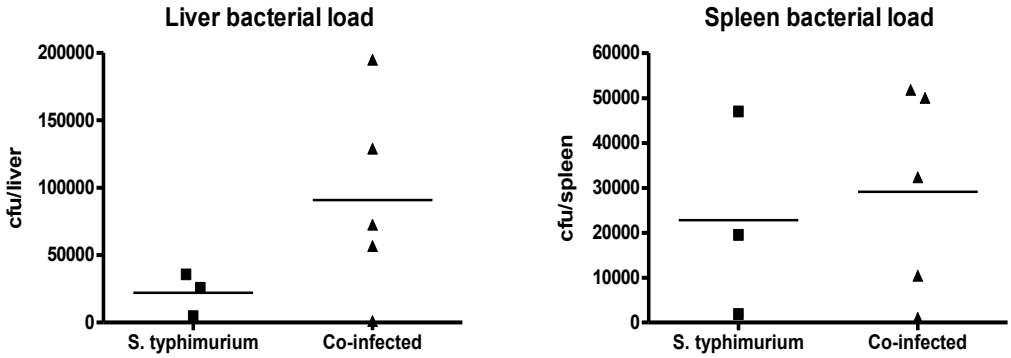
Based on epidemiological observations suggesting an association between schistosomes and other pathogens, we hypothesized that a regulatory environment established by schistosome infection would increase host susceptibility to co-infection, especially with infections that are controlled by Th1 responses. Schistosome-salmonella co-infection has been shown to result in a

prolonged salmonella infection, perhaps due to the physical association of salmonella bacteria with the schistosome worm [21] and as a result, concomitant treatment for both infections has been recommended rather than treating for either infection alone [22]. Understanding the immune alterations involved in this protracted salmonella infection is important for residents of co-endemic communities. We therefore performed a co-infection in mice with *S. mansoni* and *S. typhimurium* to determine whether regulation of the host immune response by schistosome worms would allow the salmonella bacteria to grow to higher levels than in a host singly infected with *S. typhimurium.* Bacterial load was analyzed by measuring colony forming units (cfu) in the liver and spleen of mice infected only with salmonella and compared to bacterial load in mice that were first infected with a pre-patent schistosome infection, and then co-infected with salmonella. There was an increase in the bacterial load in the liver of co-infected mice as compared to salmonella only infected mice, although this difference did not reach statistical significance, and there was a slight increase in bacterial load in the spleen of co-infected mice (Fig. 18). These data suggest that there is a physiological relevance to the regulatory milieu that is induced by pre-patent schistosome infection and that this regulatory response may have implications for increased host susceptibility to co-infection. The mechanism responsible for this increase in susceptibility may involve alterations in cell-mediated immunity that is required to control salmonella infection. This type of mechanism has been implicated in schistosome co-infection with an intestinal nematode, where an increase in alternatively activated macrophages (AAM) and

Tregs is associated with the prevention of Th1 and Th17 cell-mediated inflammation, skewing the immune response towards a Th2 phenotype [23]. Schistosome infection may up-regulate AAMs and Tregs, leading to a down-regulation of Th1 cell-mediated immunity which is essential for controlling salmonella infection. Further substantiation is found in a co-infection model demonstrating that helminth infection induces AAMs that are impaired in their ability to kill bacteria, leading to an increase in the load of an enteric bacterial infection [24]

Figure 18. Co-infection with *Schistosoma mansoni* and *Salmonella typhimurium* increases bacterial load

C3H/HeN wildtype mice were infected percutaneously with 150 *S. mansoni* cercaria, then three weeks later infected by gavage with 10^6 colony forming units (cfu) of *S. typhimurium*. Control groups included mice with *S. mansoni* only, *S. typhimurium* only, or non-infected mice. At four weeks post *S. mansoni* infection, mice were sacrificed and bacterial load was assessed in the spleen and liver by calculating cfu per organ. Horizontal bars represent the mean of 5 independent mice.


Liver bacterial load
cfu/liver
200000
150000
100000
50000
0
S. typhimurium
Co-infected
Spleen bacterial load
cfu/spleen
60000
50000
40000
30000
20000
10000
0
S. typhimurium
Co-infected

Immune Response to Schistosomiasis

Manipulation of Regulatory Response for Therapeutics

One aspect of schistosome infection, and more generally parasitic infection at large, which makes the discovery of new drugs and vaccine strategies so difficult, is that schistosomes have developed alongside their mammalian hosts for millions of years [25]. Importantly, schistosomes have developed in conjunction with a vigorous host immune response. It has been eluded that this co-evolution has led schistosomes to expand their ability to evade the host's immune system [26]. These mechanisms of evasion may in fact contribute to the persistence of schistosomes in the host and facilitate their extended survival [27, 28]. The therapeutic manipulation of parasite mediated immune modulation could be an alternative strategy for combating schistosomiasis. There is precedence for this form of treatment in not only the field of infectious disease [29], but also in the area of cancer immunotherapy [30, 31] and in the management of auto-immunity [32]. More specifically, one study examined the enhancement of T cell responses to parasitic infection in an attempt to increase immunity to the parasite [33]. Harnessing the ability of parasitic infections to modulate the host's immune response and manipulating this immuno-modulation to protect the host may be an option in treating diseases associated with parasitic infection.

The goal of our investigations was founded in the assertion that parasites are capable of regulating the host's immune response for their own advantage,

and that examining this immuno-modulation could be beneficial for the development of new treatment methods. These investigations were focused on the immune response to pre-patent schistosome infection. The immune response to schistosome infection includes an early phase that occurs while the juvenile schistosome worm is developing and a later phase that is mounted in response to egg deposition, once the worms have matured sexually and paired [34]. The schistosome egg induced immune response has been extensively studied and is known to be governed by a strong Th2 type response [35] that over time becomes down-modulated [36] and is maintained throughout chronic infection. The egg induced Th2 response leads to the inhibition of Th1 responses with the production of the regulatory cytokine IL-10 [37, 38]. IL-10 is known to control bothTh2 and Th1 responses that are in excess during schistosome infection [39]. There is a delicate balance between Th2 and Th1 responses which must be maintained during schistosomiasis to prevent immune mediated damage to the host.

Th17 Type Responses

The down-regulation of the Th1 response that occurs in an egg producing schistosome infection is coupled by an increase in Th2 responses [40], and as IFN-γ is a potent inhibitor of Th17 responses [41] may also be accompanied by an increase in Th17 responses. IL-17 is a pro-inflammatory cytokine that is characteristically produced by $CD4^+$ T cells during Th17 responses and functions

to counter regulate the inflammatory cytokine IFN-γ [42]. The cytokines that stimulate the differentiation of naïve T cells to Th17 type cells include IL-23, IL-6 and TGF-β, a collection that is very similar to the stimulation required for the differentiation of Treg cells, where both share the requirement for TGF-β [43, 44]. Th17 development is associated with IL-23, a member of the IL-12 family of cytokines, which shares a common IL-12p40 sub-unit with IL-12 [45]. Th17 and Th1 responses are closely associated because of this shared cytokine sub-unit that is part of the cytokine stimulus for both responses. The counter-regulation that occurs between Th1 and Th17 responses is critical for the development of egg-induced pathology [46]. In the absence of IFN-γ in schistosome infection, there is a marked increase in immunopathology due to schistosome eggs and an increase in IL-17. However, in the absence of IL-17 this immunopathology is significantly reduced due to increased IFN-γ [47].

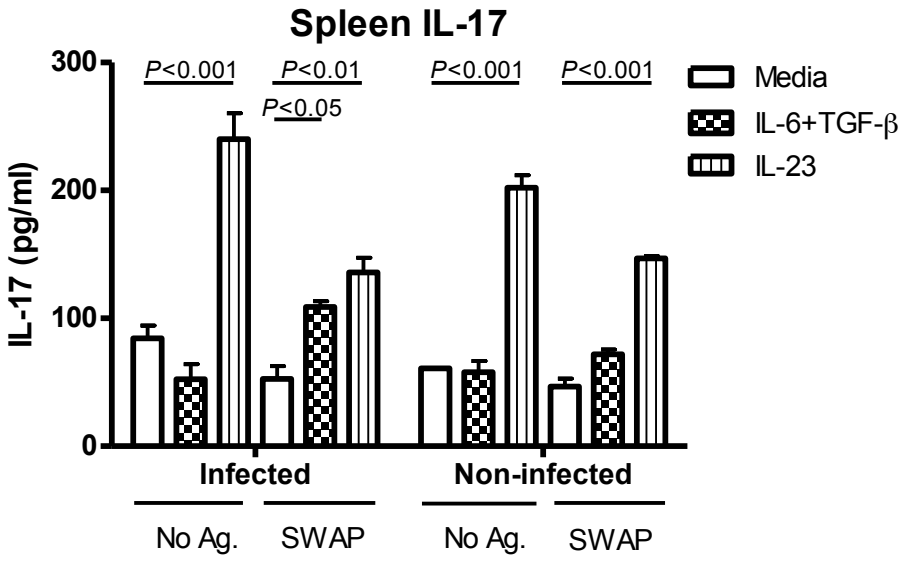
IL-17 in response to pre-patent schistosome infection

Although Th17 type responses seem to have a role during patent schistosome infection, our data indicate that IL-17 is not produced in response to pre-patent schistosome infection. We were able to elicit the production of IL-17 by T cells isolated from the spleen of pre-patent schistosome infected and non-infected mice, using cytokine conditions that have been shown to induce Th17 responses [48]; specifically IL-6 plus TGF-β or IL-23. IL-23 stimulation of cells from infected or non-infected mice elicited a significant increase in IL-17

production, in both cases where the cells received no additional antigenic stimulation or where the cells were also stimulated with SWAP. However, there was only a slight increase in IL-17 production when cells from infected or non-infected mice were stimulated with IL-6 plus TGF-β, in addition to SWAP stimulation. There was no difference in SWAP stimulated production of IL-17 from infected mice compared to non-infected mice with either IL-17 inducing stimulation (Fig. 19). These data suggest that there is not a significant induction of Th17 responses, with the production of IL-17, in response to pre-patent schistosome infection. SEA stimulated IL-17 production has been shown to be initiated by IL-23 and, interestingly, amplified by IL-1β [49]. These findings correlate with our results showing that IL-1β expression is down-regulated in splenic tissue in response to pre-patent schistosome infection (Fig. 9B), and so IL-17 production would not likely be stimulated under these conditions.

Figure 19. Pre-patent schistosome infection does not induce IL-17 production

Splenocytes from four week *S. mansoni* infected wildtype mice or non-infected mice were cultured with no antigen or SWAP stimulation, and additionally IL-6 plus anti-TGF-β, or IL-23. Cells were cultured for three days and production of IL-17 in culture supernatant was analyzed by ELISA. Data is represented as mean +/- SEM. SWAP, soluble worm antigen preparation; αTGF-β, anti-TGF-β.


Spleen IL-17
IL-17 (pg/ml)
300
200
100
0
P<0.001
P<0.01
P<0.05
P<0.001
P<0.001
Media
IL-6+TGF-β
IL-23
Infected
Non-infected
No Ag.
SWAP
No Ag.
SWAP

Immuno-regulation

The pre-patent immune response to schistosome worms has not been studied as extensively as the response to schistosome eggs. It has been characterized as a weak Th1 response [50] and has also been shown to include Th2 responses to worm antigen [51]. We have presented evidence that this early immune response additionally includes regulatory responses, with the expression of the regulatory cytokine IL-10 and production of IL-10 by $CD4^+$ T cells in response to worm antigen (Ferragine, Chapter 2). Understanding the innate immune response to pre-patent schistosome infection is critical as these responses are the foundation for the adaptive response that develops upon egg deposition. The scale and quality of the adaptive immune response that forms as a consequence of the innate immune response is crucial in the control of egg induced pathology. The relevance of the pre-patent immune response to schistosome worms is emphasized by appreciating the significance of the balance between host protective Th2 responses, which sequester the schistosome egg, and potentially detrimental inflammatory responses which can damage host tissue. Immuno-regulation functions to suppress excessive immune responses and maintain immune homeostasis. The role of IL-10 in immuno-regulation is especially relevant in schistosomiasis, as IL-10 has been implicated in the control of Th1 responses during acute and chronic schistosomiasis in human patients [52].

Immuno-regulation can be achieved by several different mechanisms. One such mechanism is the induction of an immunologically hyporesponsive

state. Immunological hyporesponsiveness has been associated broadly with chronic infections [53] and more specifically with helminth infections [54, 55] and may include the modulation of both innate and adaptive immune responses [56-58]. The induction of this hyporesponsive state in the T cell compartment can be caused by a variety of mechanisms. T cell anergy is a form of tolerance where T cells become hyporesponsive and are functionally inactive after an encounter with antigen, but can survive for prolonged periods of time [59]. Th2 cell responses have been shown to become hyporesponsive during chronic schistosomiasis and have been demonstrated to occur via a cell intrinsic mechanism of tolerance [60]. Interestingly, another cell intrinsic mechanism of tolerance has been identified during schistosomiasis, where IL-10 has been implicated in the induction of $CD4^+$ T cell anergy [38]. Other mechanisms that may lead to $CD4^+$ T cell hyporesponsiveness during schistosomiasis include the stimulation of $CD8^+$ [61] or $CD4^+$ [62] regulatory T cells. These forms of T cell-mediated suppression are considered to be cell extrinsic mechanisms of tolerance, whereby certain populations of T cells function to maintain the proper activation and expansion of other populations of lymphocytes [63]. The identification of these various forms of tolerance during patent schistosome infection reinforces the critical role of immuno-regulation in the control of immune mediated pathology.

Our studies were first aimed at the examination of the immune response that develops to pre-patent schistosome infection; to determine whether an immunologically hyporesponsive state is induced by schistosome worms.

Previous studies investigating the immune response during a patent schistosome infection of wild type mice have shown a reduction in IFN-γ production in response to polyclonal stimulation [64]. Our findings are the first to suggest that T cell hyporesponsiveness is associated with pre-patent infection, evidenced by a significant impairment in production of IFN-γ and $CD4^+$ T cell proliferation in response to polyclonal stimulation and the specific antigen OVA. The induction of a regulatory response to schistosome worms may have a critical role in the hyporesponsive T cell phenotype that is induced by pre-patent infection. In fact, previous data from our lab indicated that the production of IFN-γ is unrestrained in IL-10 knockout mice in response to schistosome infection, as compared to infected control mice (data not shown). These data implicate IL-10 as an essential inhibitor of Th1 responses and the production of IFN-γ. As mentioned previously, we have shown that IL-10 is expressed and produced by $CD4^+$ T cells during pre-patent infection in an egg-independent manner. Prior to our work, it was not clear whether a regulatory response is induced by schistosome worm infection, and additionally whether the production of IL-10 is a crucial component of this regulatory response.

To determine whether Tregs play an essential role in the immune regulation that is induced by schistosome worms, we used two different mechanisms to impair the population of nTregs in vivo. IL-2 signaling, which is critical for the function and maintenance of nTregs [65], was inhibited or the population of $CD25^+$ cells was depleted in vivo. Although we did achieve significant depletion of the $CD4^+$ $CD25^+FOXP3^+$ nTreg population with both

approaches, there was no significant alteration in the production of cytokines in response to worm antigen. These findings suggest that nTregs are not a significant source of IL-10 during pre-patent schistosome infection and that they are also not the population of cells that is essential in restricting the production of IFN-γ.

To determine whether the loss of $CD4^+$ T cell responsiveness and IFN-γ production that we see upon polyclonal stimulation also includes a defect in T cell responses to non-schistosome worm antigens, we used the OT-II/RAG-$1^{-/-}$ mouse model. These mice lack all B and T cells except for a monospecific population of T cells which only recognize the specific $OVA_{323-339}$ peptide. This model allowed us to exclude the role of $CD4^+$ T cells that respond to schistosome infection, including IL-10-producing $CD4^+$ T cells. When splenocytes from infected OT-II/RAG-$1^{-/-}$ mice were stimulated with OVA peptide or polyclonal stimulation, we again saw the loss of cytokine production and $CD4^+$ T cell proliferation. These findings provide evidence that there is also a loss of $CD4^+$ T cell responsiveness to non-parasite antigens in the context of a schistosome infection and that this hyporesponsive state is independent of an adaptive immune system response to schistosomes. Our lab has shown previously that $CD4^+$ T cells isolated from OT-II/RAG-$1^{-/-}$ mice are fully capable of responding to OVA stimulation and that there is no difference in the production of IFN-γ by OT-II T cells from infected of non-infected mice [66]. Together, these findings suggest that the loss of $CD4^+$ T cell responsiveness is a result of alterations in the innate APC population, as opposed to a direct effect on T cells. We were

able to confirm the importance of innate immune cells in modulating T cell responsiveness to pre-patent schistosome infection by showing that the inhibition of cytokine production and $CD4^+$ T cell proliferation additionally occurs when splenocytes from infected RAG-$1^{-/-}$ mice were used as APCs and co-cultured with OT-II $CD4^+$ T cells.

Innate Antigen Presenting Cells

The mononuclear phagocyte system consists of several subsets of cells; including dendritic cells and mononuclear phagocytes [67], many of which have been implicated in the control of T cell responses [68]. Gr-1^+ cells, which may include populations of neutrophils, dendritic cells, and monocytes [69], have been shown to have an immuno-regulatory role in impairing T cell responses [70, 71]. We chose to investigate $CD11c^+$ dendritic cells, as dendritic cells are known to have a crucial role in priming Th2 responses during schistosome infection [72, 73]. Macrophages have been shown to have a immune modulatory role during schistosome infection by inducing T cell anergy [74] and type 2 differentiation during schistosomiasis [75]. Based on these findings, we also investigated the role of $CD11b^+$ mononuclear cells in T cell hyporesponsiveness induced by schistosome infection.

Helminths as a group are known to modulate innate immune cell function. One example of this modulation is the impairment of antigen processing and presentation by cystatin, a protease inhibitor found in helminth ES products [76].

Cystatins have also been shown to reduce the expression of co-stimulatory molecules on APCs and impair T cell proliferation [77]. Our phenotypic analyses of $CD11c^+$ and $CD11b^+$ cell populations investigated whether schistosome infection causes an alteration in the expression of MHC class II molecules or co-stimulatory ligands, both of which are required for effective $CD4^+$ T cell stimulation by APCs [78-80]. A reduction in the expression of these molecules at the point of T cell priming could lead to T cell hyporesponsiveness [81]. However, our data show no evidence that MHC class II or the co-stimulatory marker CD86 are down-regulated by schistosome infection. In fact, both molecules are up-regulated in response to schistosome infection on populations of $CD11c^+$ dendritic cells and $CD11b^+$ mononuclear cells. Functional analyses of the ability of innate APCs to stimulate $CD4^+$ T cell responses during schistosome infection showed that $CD11c^+$ dendritic cells are fully capable of stimulating naïve $CD4^+$ T cells. However, $CD11b^+$ cells were impaired in their ability to stimulate IFN-γ production and OT-II T cell proliferation. These findings correlate with a previously identified regulatory role for macrophages in the context of a schistosome infection.

We next sought to identify the mechanism by which $CD11b^+$ mononuclear cells that have been characteristically modified by schistosome infection cause impairment in $CD4^+$ T cell responsiveness. Our hypothesis was that pre-patent schistosome infection induces innate APCs to act as suppressor cells, which is consistent with previous studies showing that a schistosome-expressed polysaccharide has immunomodulatory properties and elicits a population of

suppressor macrophages that are able to inhibit T cell proliferation [82]. We used splenocytes from infected RAG-1$^{-/-}$ mice as a source of APCs in co-culture with OT-II CD4^{+} T cells to test this hypothesis. Inhibitors were added to these co-cultures in an attempt to restore proliferation back to levels seen when splenocytes from non-infected RAG-1$^{-/-}$ mice are used as APCs. We targeted several known regulatory mechanisms based on their role in the suppression of T cell proliferation by MDSCs [83], to further examine how T cell responsiveness is impaired by schistosome infection. Arginase and nitric oxide synthase are enzymes that have a role in the inhibition of T cell proliferation by MDSCs [83]. The regulatory cytokine IL-10 is known to inhibit the priming of T cell responses by antigen presenting cells [84] and another regulatory cytokine, TGF-β, produced by macrophages, has been shown to suppress T cell responses in schistosome infection [85]. The molecule PD-L1, expressed on macrophages, has been shown to induce anergy in T cells in response to schistosome infection [74]. However, our experiments inhibiting these regulatory mechanisms did not lead to restoration of proliferation in co-cultures of splenocytes from infected RAG-1$^{-/-}$ mice and OT-II CD4^{+} T cells.

Additionally, our data do not indicate a role for the induction of T cell anergy in schistosome infection. OT-II T cells from infected mice were shown to be fully capable of responding to OVA stimulation by producing IFN-γ [66]. The provision of exogenous IL-2 is known to reverse T cell anergy and re-establish proliferation [86], but we were unable to restore proliferation of OT-II T cells in co-cultures with splenocytes from infected RAG-1$^{-/-}$ mice by supplementing with

exogenous IL-2. This result further supports our conclusion that a general anergic state is not induced in T cells in response to schistosome infection.

Interfering with various regulatory mechanisms did not provide an explanation for the reduction in T cell stimulatory capacity by schistosome infection. We next hypothesized that the impairment was cell-contact dependent, which is consistent with our findings that T cell suppression is not mediated by diffusible factors such as IL-10 and TGF-β. We observed that the impairment of T cell responses is in fact contact-dependent and only occurs when T cells are directly mixed with a source of APCs from infected mice. Additionally, APCs from infected mice need to outnumber competent APCs before suppression of T cell proliferation is seen. These data indicate that the phenotype induced in APCs by schistosome infection is not that of a strong suppressor cell, but is more likely a phenotype of impaired capacity to function as antigen presenters and stimulate T cells. It may be that infected APCs outcompete competent APCs for T cell contact and so T cells are not effectively stimulated.

Our findings demonstrating the down-regulation of IL-12 expression in response to schistosome infection further support the hypothesis that infection causes APCs to have a reduced T cell stimulatory capacity. Schistosome infection appears to cause a defect in the expression of IL-12 by APCs which may impair their ability to stimulate the production of IFN-γ by T cells. Providing exogenous IL-12 to co-cultures with splenocytes from infected mice used as APCs restored production of IFN-γ, but did not restore T cell proliferation. These findings indicate that schistosome infection causes a reduction in the production

of IL-12 by APCs, but this is not likely the only mechanism that is functioning to impair T cell responsiveness and it is likely a combination of mechanisms. It may be that the mechanisms which were tested are redundant and need to be inhibited in combination in order to see restoration of T cell responses. Additionally, resistin-like molecule (RELM)-alpha and the induction of an AAM phenotype has been implicated in the regulation of $CD4^+$ T cell responses by schistosome infection [87] and may have a role in the impairment of T cell responsiveness.

Implications of Findings

IL-12 Deficiency and Regulatory Responses

There are some important implications to the induction of a defect in IL-12 production in response to pre-patent schistosome infection. A form of immunologic paralysis occurs in dendritic cells that have responded to infection by producing IL-12, but then are no longer able produce IL-12 in response to continued stimulation [88]. This may be a mechanism of inhibiting immuno-pathological effects that could be mediated by prolonged exposure to the cytokine. It may also be a normal process that allows the host to initiate an immune response to infection and develop cell-mediated immunity. This mechanism is thought to be an important means of protecting the host and would also be relevant in schistosomiasis as a method to shield the host from egg-induced immuno-pathology. IL-12 deficiency in response to schistosome

infection may also render the host more susceptible to co-infection with pathogens that are controlled by pro-inflammatory responses and IFN-γ production. The induction of a regulatory response may also be important in increasing host susceptibility to co-infection; again with co-infecting pathogens that elicit Th1 responses. These schistosome-induced regulatory responses are important in the context of therapeutic development for schistosomiasis. The identification of an essential suppressive or inhibitory molecule could lead to the development of new drugs or vaccine strategies for the treatment of schistosomiasis.

Future Directions

This work serves to provide an investigation of the immune response to pre-patent schistosome infection in the broader context of the how the modulation of the host's immune response facilitates the schistosome worm's persistence. We have shown that schistosome infection impairs T cell responsiveness, which may be a method that facilitates schistosome survival by impairing the development of an effective adaptive response that would be capable of eliminating the parasite. We propose a model whereby schistosome worms induce alterations in the innate APC population which impair their ability to act as antigen presenting cells and stimulate T cell responses. A deficiency in IL-12 production by innate APCs in response to pre-patent schistosome infection is one mechanism that is implicated in the impairment of T cell stimulatory

capacity. This impairment of T cell responsiveness, and the loss of a pro-inflammatory response, may be responsible for allowing the development of the schistosome worm in the host. Further investigation is required to determine how schistosome worms modulate the innate immune response in such a way to impair T cell responsiveness and how this relates to parasite persistence. It is likely that a variety of mechanisms are functioning during this impairment and future studies will need to examine the methods by which pre-patent schistosome infection induces this impairment in the T cell stimulatory capacity of innate APCs.

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